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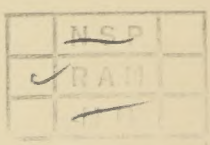

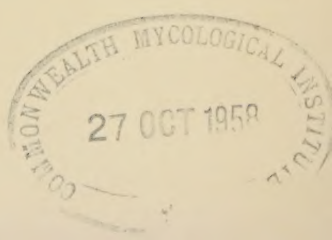

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MICROBIOLOGICAL HYDROXYLATION OF STEROIDS I. MICROBIOLOGICAL HYDROXYLATION OF PROGESTERONE BY *RHIZOPUS*

TOSHINOBU ASAI, KYOSUKE TSUDA, KŌ AIDA, EIJI OHKI,
TOKUJI TANAKA, MASAKO HATTORI and
HARUO MACHIDA

The Institute of Applied Microbiology, and Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

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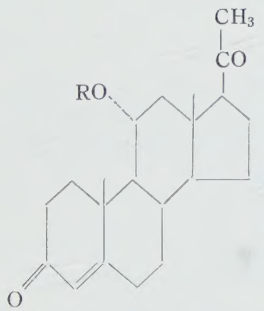
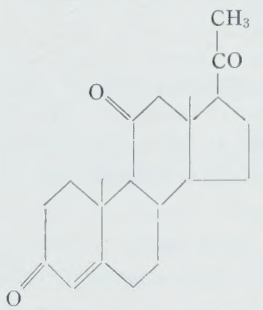
In recent years, a new synthetic process[1] to introducing a substituent into the steroid skeleton by means of enzymatic hydroxylation of microorganisms for the preparation of adrenocortical hormones whose chemical synthesis would otherwise be extremely difficult and require a long process has been established. It has now become possible to introduce a hydroxyl group into the 11-position of steroids such as progesterone, cortexone, and compound S to form 11-hydroxy- and 11-oxocorticostroids by the use of microorganisms such as *Rhizopus*[2] and *Aspergillus*[3].

Before going into this study, the capability of such hydroxylation was tested with 284 strains of *Rhizopus* sp. preserved in this Institute, using progesterone. As it will be described in the experimental section, the microorganism was incubated, the fermentation liquor was extracted, and the concentrated extract examined by paper chromatography, following the method of Zaffaroni[4]. It was thereby found the 21 strains of this species, listed in Table 1, had the ability to effect this hydroxylation. Further, mass-culture was carried out on a few strains, the products isolated and purified



(I) R=H
(II) R=$COCH_3$



(III)

by chromatography, upon which hydroxylation product was identified to be 11α-hydroxyprogesterone (I). Its physical constants, its acetate (II), and formation of 11-oxoprogesterone (III) by further oxidation, all agreed well with those reported in previous literature[5].

Table 1 Hydroxylation of Progesterone by *Rhizopus* sp.

Species	No.	Hydro-xylation ability	Product
Rh. nigricans YAMAZAKI	R5- 4	+++	11α-Hydroxyprogesterone (identified)
Rh. 〃 〃	R7- 7	++	〃
Rh. chinensis SAITO	10-10	+++	〃
Rh. chiuniang YAMAZAKI	14-14b	++	〃
Rh. arrhizus FISCHER	R5- 6	++	〃
Rh. Kansho YAMAMOTO	28-14	++	〃
Rh. nigricans EHRENBERG	37-12	+	11α-Hydroxyprogesterone (by paperchromatography)
Rh. 〃 〃	37-27	++	〃
Rh. delemar WEHMER et HANZAWA	19-27	+	〃
Rh. formosensis NAKAZAWA	21-29c	+	〃
Rh. japonicus VUILLEMIN	24-14	+	〃
Rh. 〃 〃	24-14b	+	〃
Rh. javanicus TAKEDA	26-23	+	〃
Rh. nodosus NAMYSLOWSKI	39-29a	+	〃
Rh. pseudochinensis YAMAZAKI	48-29c	+	〃
Rh. tonkinensis VUILLEMIN	65-10	++	〃
Rh. tritici SAITO	66- 5a	+	〃
Rh. 〃 〃	66-14c	+	〃
Rh. 〃 〃	66-27	+	〃
Rh. niveus YAMAZAKI	R5- 5	+	〃
Rh. oryzae WENT et PRINSEN-GEERLIGS	R5- 7	+	〃

The authors wish to express their gratude to Mr. K. Aizawa, Dept. of Agricultural Chemistry, University of Tokyo, for infrared spectral measurements, and to Miss H. Yamanouchi and Mr. Y. Satoh of the Microanalysis Divison of this Institute for elemental analysis.

EXPERIMENTAL

1. Examination of the Hydroxylation Ability of Each Strain.

The medium used here for selection of the strain contained 5% glucose, 2% peptone, and 0.3% corn steep liquor. Ten ml of this medium was placed in each test tube of 35 ml capacity, (17×240 mm) previously cotton plugged and

sterilized, and sterilized at 15 lb for 10 minutes (pH 5.2). The seed cultured on Koji agar for 2 weeks at 28°C was inoculated, test tubes were stood in a rack, and submitted to reciprocal shake culture (120 r.p.m.) at 30°C. The test tubes were then slanted at 45°. Forty-eight hours after inoculation, a solution of 2 mg of progesterone dissolved in 0.5 ml of ethanol was added and culture was continued further for 48 hours (pH 4.0–4.2). To each test tube, 10 ml of ethyl acetate was added, extracted for 3 minutes in a homogenizer, and the whole solution was filtered by suction to remove the cells. The ethyl acetate layer was transferred to a small beaker and concentrated under a reduced pressure.

Subsequently the concentrated residue was submitted to paper chromatography following the hydrazone method of Zaffaroni[4] to examine the presence of spots other than that of progesterone. Paper chromatography conducted by the ascending technique using a water-butanol mixture should give a spot of progesterone with the Rf value of 0.13–0.17 but the products obtained from culture of microorganisms listed in Table 1 gave one spot at Rf 0.06–0.10, which agreed with the spot of 11α-hydroxyprogesterone described below.

2. Isolation and Identification of 11α-hydroxyprogesterone.

An example of experiments with *Rhizopus chinensis* Saito will be given. Experiments conducted with others followed a similar procedure.

One liter of medium containing 5% glucose, 2% peptone, and 0.3% corn steep liquor was prepared and 100 ml of this medium was placed in each sterilized shaking flask of 500-ml capacity. The flasks were sterilized for 15 minutes at 15 lb., cooled (pH 5.2), and one loop of mycelium grown on Koji agar slant was inoculated. The flasks were submitted to reciprocal shake culture (120 r.p.m.) for 48 hours at 30°C. A solution of 50 mg of progesterone dissolved in 2 ml of acetone was added for each 100 ml of the medium. The total amount of progesterone used was 0.5 g. The flasks were further incubated for 48 hours (pH 4.0 at the end of culture).

The content of the flask was filtered, cells were homogenized twice with 150-ml portions of acetone, filtered, and the acetone extract was combined with the fermentation broth. The combined liquor was extracted twice with 800-ml portions of ethyl acetate, the acetate extract washed twice with 2% sodium bicarbonate solution, twice with water, and concentrated. The yield of the concentrated extract was 1.05 g.

This concentrated extract was submitted to column chromatography, using 40 g of alumina. From the portion eluted with benzene and benzene-ether (7 : 3), 65 mg of progesterone, m.p. 128°, was recovered. The chloroform eluate afforded 0.251 g of crystals, m.p. 154–157°. Yield, 50%. Recrystallization from ether-chloroform gave 11α-hydroxyprogesterone (I), m.p. 168.5–169.5°.

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15.
Found: C, 76.05; H, 9.22.

$[\alpha]_D^{20}+178°$ (c=1.4, $CHCl_3$).

U.V. λ_{max}^{EtOH} 241.3 mμ (ε 14100).

I.R. $\lambda_{max}^{CHCl_3}$ 3450 (OH), 1700 (20–CO), 1670(3–CO), 1610 (Δ^4) cm^{-1}

This substance was acetylated with acetic anhydride and pyridine at room temperature and its acetate (II), m.p. 174.5–174.6°, $[\alpha]_D^{20}$ +127° (c=0.78, acetone), was obtained.

To a solution of 31 mg of this substance dissolved in 1 ml of glacial acetic acid, glacial acetic acid solution of 7 mg of chromium trioxide was added, the mixture then allowed to stand at room temperature for 2 hours, diluted with water, and extracted with chloroform. Treatment of the neutral portion afforded 21 mg of crystals which were recrystallized from methanol and confirmed to be 11-oxoprogesterone (III), m. p. 171–176°, $[\alpha]_D^{20}$ +220° (c=1, $CHCl_3$).

Anal. Calcd. for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59.
Found: C, 76.60; H, 8.64.

SUMMARY

Oxidizability of progesterone was examined with 284 strains of *Rhizopus* sp. preserved in this Institute. Among these, 21 strains were found to have such ability. Mass culture of five of these strains was carried out and the oxidation product was identified as 11α-hydroxyprogesterone. The yield obtained was approximately 50%, using *Rh. chinensis* SAITO 10–10.

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MICROBIOLOGICAL HYDROXYLATION OF STEROIDS II. MICROBIOLOGICAL HYDROXYLATION OF PROGESTERONE BY ASPERGILLUS

HIROSHI IIZUKA, ATSUSHI NAITO and MASAKO HATTORI

Division of Type Culture Collection, Institute of Applied Microbiology, University of Tokyo

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INTRODUCTION

Although various kinds of oxygenated steroids had been synthesized through complicated processes of organic synthetic reactions in the post, Fried, et al. in 1952, followed by Murray and Peterson in 1953, found that a hydroxyl group is easily introduced into the 11α-position of progesterone and Reichstein's compound S (11-desoxy-17-hydroxycorticosterone) by *Aspergillus niger*. Thereforth, studies on microbiological hydroxylation of steroids have made a marked progress.

The present authors[14] compared oxygenation ability of 473 strains of *Aspergillus* preserved in this Institute and have selected some useful strains. Examinations were carried out on the culture conditions of the strains selected on hydroxylation of steroids, and isolation and identification of the product were made by the application of tank culture.

EXPERIMENTAL

1. Comparison and Selection of Aspergillus Strains

A. Sample Strains

A total of 473 strains were submitted to the test. These included 182 strains of representative *Aspergillus* sp. preserved in this Institute, 243 strains of Black-Koji molds[1], and 48 strains of induced mutants obtained by ultraviolet irradiation by one of the authors[2-4]. Names of species and strains are listed in Tables 1, 2, and 3.

B. Experimental Method[5]

Medium: The medium consisted of 2% peptone, 5% glucose, and 0.3% corn steep liquor, pH 5.4. Ten ml of this medium was placed in a large test tube (2×20 cm) and sterilized for 10 minutes at 15 lbs (120°C). Fermentation method: The fungal strain was inoculated on the medium thus prepared and submitted to shake culture (118 r.p.m.) for 48 hours at 30°C.

A solution of 1 mg of progesterone contained in 1 ml of ethanol was then added and the mixture was incubated for 48 hours further, under the same conditions.

Table 1. Comparison of various strains of *Aspergillus* sp.

Name of species	No. of Strain	Other than substrate		Name of species	No. of strain	Other than substrate	
		1 spot	2 spots			1 spot	2 spots
A. clavatus	2	2		*A. quadrilineatus*	2	1	
A. giganteus	2	1		*A. rugulosus*	2	1	
A. amstelodami	1			*A. unguis*	1		
A. caesiellus	1			*A. variecolor*	1		
A. chevalieri	1			*A. ustus*	11	1	
A. echinulatus	2	1		*A. flavipes*	1	1	
A. glaucus	1			*A. janus*	1		
A. gracilis	1	1		*A. sydowi*	4	2	
A. herbariorum	1			*A. versicolor*	4	2	1
A. itaconicus	1	1		*A. carneus*	1	1	
A. minimus	1	1		*A. terreus*	4		1
A. mollis	1	1		*A. candidus*	1	1	
A. proliferans	1			*A. carbonarius*	1		
A. pseudoglaucus	1			*A. cinnamomeus*	1	1	
A. repens	3	3		*A. fonsecaeus*	1		
A. ruber	1	1		*A. japonicus*	3	2	
A. varians	2	1	1	*A. miyakoensis*	2	1	1
A. fischeri	4	4		*A. niger*	26	12	7
A. fumigatus	8	4		*A. phoenicis*	1		
A. caespitosus	1			*A. pulverulentus*	1		
A. nidulans	6	3		*A. violaceo-fuscus*	1		1
A. alliaceus	1	1		*A. avenaceus*	1		
A. panamensis	1			*A. wentii*	6		2
A. citrisporus	1		1	*A. tamarii*	13	5	
A. terricola	2		1	*A. flavus*	2	1	
A. gymnosardae	1			*A. oryzae*	16	1	
A. parasiticus	2	1		*A. sojae*	3	2	
A. butyracea	1			*A. auricomus*	1		
A. melleus	1			*A. elegans*	2	2	
A. ostianus	1		1	*A. ochraceus*	5	2	1
A. sclerotiorum	2			*A. rehmii*	1		
A. sulphureus	1	1		*A. sparsus*	1		
A. asperescens	1	1		*A. aculeatus*[15]	1		
A. brevipes	2	1		*A. kanagawaensis*	1		

Total 182 strains; oxygenation ability, 83 strains (45.6%).

Estimation of the oxygenation product: Ethyl acetate was used as the extraction solvent and the extraction residue was submitted to paper chromatography, applying the hydrazone method of Zaffaroni[6],[11-13] adopting the ascending technique. The products identified were 11α-hydroxyprogesterone and 6β, 11α-dihydroxyprogesterone.

C. Experimental Results

Results of paper chromatographic experiments are given in Tables 1, 2, and 3.

Table 2. Comparison of various strains of Black-Koji Molds.[1]

Name of species	No. of strain	Other than substrate		Name of species	No. of strain	Other than substrate	
		1 spot	2 spots			1 spot	2 spots
A. usamii	12	8	3	*A. saitoi*	68	6	
A. inuii	20	5	1	*A. batatae*	1		
A. aureus and varieties	24	1	3	*A. nakazawai*	18	2	1
				A. kawachii	1		
A. awamori and varieties	99	20	7				
Total 243 strains; oxygenation ability, 59 strains (24.3%)							

Table 3. Comparison of induced mutants of *Aspergillus*.[4]

Name of species	No. of strain	Other than substrate		Name of species	No. of strain	Other than substrate	
		1 spot	2 spots			1 spot	2 spots
A. usamii U.V. mutant	33	8		*A. wentii* albino mutant	1		
A. usamii 2, 4-D-mutant	5	3		*A. candidus* albino mutant	1		
A. fumigatus albino mutant	1			*A. terreus* albino mutant	1		
A. ochraceus albino mutant	1	1		*A. tamarii* albino mutant	1		
A. giganteus albino mutant	1			*A. usamii* albino mutant	1		
A. oryzae albino mutant	1			*A. sojae* albino mutant	1		
Total 48 strains; oxygenation ability, 12 strains (25.0%)							

Grand total (Tables 1, 2 and 3), 473 strains; oxygenation ability, 154 strains (33.0%)

Examination of oxygenation ability of all strains of *Aspergillus* revealed that some strains of black *Aspergillus* possessed stronger oxygenation ability than other strains. From the result of tests conducted with Black-Koji molds[1] and their induced mutants[2-3], the following strains were select-

ed as those having powerful ability of oxygenating steroids:

A. saitoi IAM R-1216, IAM H-0756
A. awamori IAM K-0924, IAM K-0625, IAM K-3532, IAM K-5112
A. nakazawai IAM K-2024
A. usamii IAM R-0635
A. usamii U. V. mutant IAM 59-1
A. usamii mut. shirousamii IAM B-407

2. Culture Conditions

Relationship between oxygenation ability and culture conditions was examined with *A. usamii* U.V. mutant IAM 59-1 strain, which from selective tests, was found to have the strongest ability to oxygenate steroids.

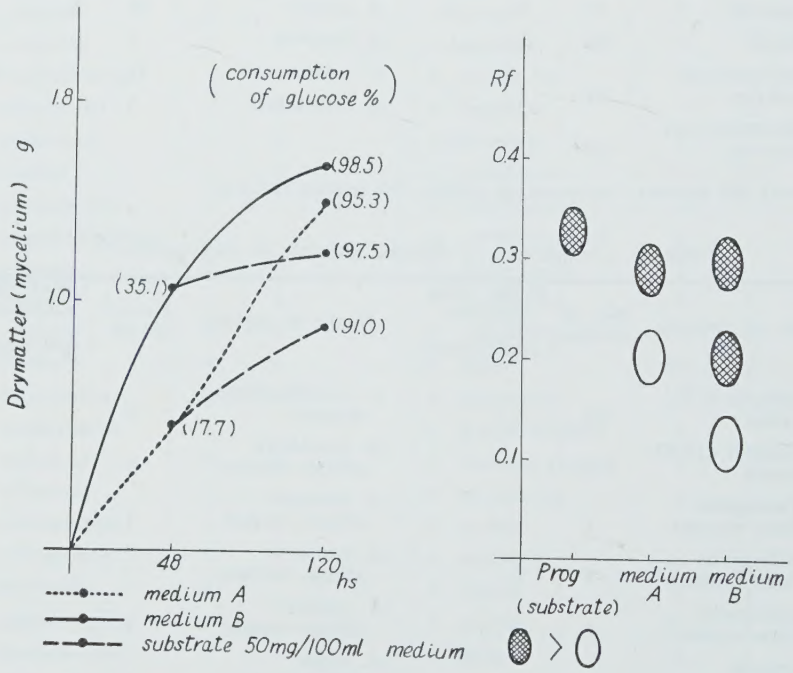


Fig. 1. Compared medium A with B on dry matter of mycelium and on paperchromatography.

A. Composition of the Medium

A number of Several different media were prepared and preliminarily tested from which media A and B, indicated below, were selected for this purpose. Comparison of the two media indicated, as it can be seen from Fig. 1 and paper chromatography, that medium B containing ammonium nitrate is more effective for the fermentation of steroids.

Medium	A	B
Peptone	2.0%	1.0%
Glucose	5.0	5.0
Corn steep liquor	0.3	0.3
Ammonium nitrate	0	0.1
pH	5.4	5.4

The prepared medium (100 ml.) was placed in a 500 ml. shake flask and sterilized for 15 minutes at 15 lbs (120°C).

Fermination: The strain of *A. usamii* U.V. mutant IAM 59–1 was inoculated on the sterilized medium, submitted to shake (118 r.p.m.) culture for 48 hours at 30°C, progesterone was then added to the medium in a ratio of 50 mg to 100 ml of the medium, and it was further incubated for 72 hours under the same conditions.

B. Effect of Solvent on Fungal Growth[7-8]

Five different solvents were used in the addition of progesterone to the medium and their effect on oxygenation ability was examined. The experimental method applied here followed that described in medium A, using medium B in this case. The results are given in Fig. 2, and an example of paper chromatography, using methanol, is shown in Fig. 3.

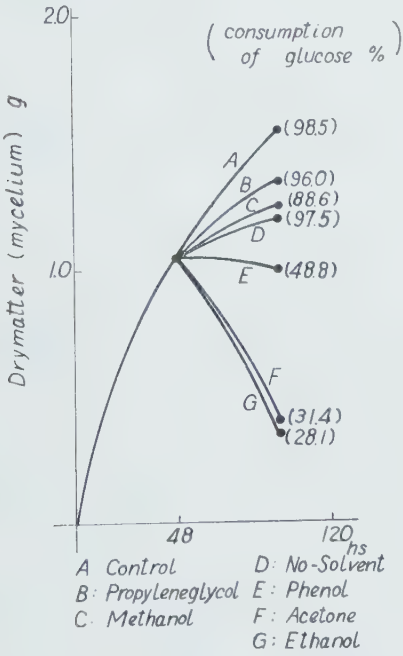


Fig. 2. Effect of various solvents on fungal growth.

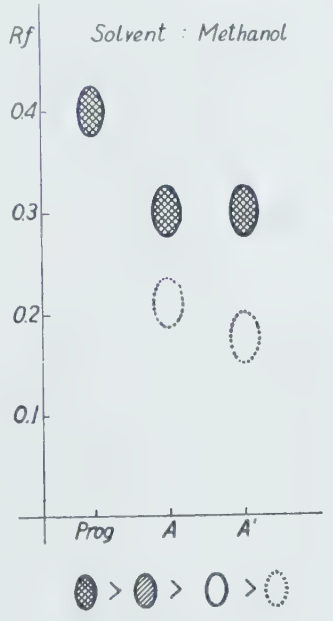


Fig. 3. An example of paper chromatograph which employed methanol.

The most suitable solvents for use in the addition of progesterone were found to be methanol and propylene glycol, while phenol, acetone, and ethanol were found to be unsuitable.

Further detailed examinations were carried out on the effect of concentrations of the solvent on fungal growth, their optimal concentration, and changes in fungal cells (cf. Table 4).

Strain used: *A. usamii* U.V. mutant IAM 59–1.

Experimental method:

Monospore $\xrightarrow[30°C]{16\text{–}17\ hrs.}$ addn. of medium containg solvent and substrate progesterone 10 mg/1 ml solvent

stood for def. period $\xrightarrow[room\ temp.]{}$ washed 5 times with water $\longrightarrow$ microscopy

$\xrightarrow[30°C]{16\text{–}17\ hrs.}$ microscopy

Indication of results

Degree of effect	Condition of cells	Degree of growth	State of growth
A	Vacuole, granular	a	No apparent growth
B	Granular	b	No observable growth
C	Slightly granular	c	Observable growth
D	No effect	d	Active proliferation

Table 4. Effect of solvents on fungal growth.

Solvent \ Time (hrs.) / %	4	5	6	7	8	9	24
Ethanol 10	A a	A a					
Ethanol 5	B b	B b	B b	A a			
Ethanol 1				D c	D d	D c	D d
Methanol 10	B a	B a	B a	A a			
Methanol 5	D d	D d	D d	D d	D d	D d	D c
Methanol 1				D c	D c	D c	D c
Acetone 10	A a	A a	A a	A a			
Acetone 5	D c	D c	C c	B b	B c	B b	B b
Acetone 1			D c	D c	D c	D b	D d
Propylene glycol 10	A a	A a	A a	A a			
Propylene glycol 5	C d	D d	D d	C c	C c	B c	B b
Propylene glycol 1						C d	D c

Solvent \ Time (min.) / %	1	3	5	30	60
Phenol 1	C a	B a	B a	A a	B a

From the above experimental results and consideration of the solubility of the solvent in the substrate and also various characteristics of the solvent shown during extraction, concentration, and crystallization, the kind of solvent and its concentration that would not impair fungal growth were as follows:

Solvent	ml/100 ml medium	Amt. of substrate mg/ml solvent
Acetone	<1	100
Methonal	<2	50

C. Period of Fermentation

Strain used: *A. saitoi* IAM R-1216
A. sp. IAM No. 28
A. usamii U.V. mutant IAM 59-1

Experimental method:

Inoculation (Medium B) → 30°C, 48 hrs., 118 r.p.m. → Addn. of substrate (progesterone 100 mg/1 ml acetone)
→ 30°C, 24 hrs., 118 r.p.m. → Sample A and A′

Inoculation (Medium B) → 30°C, 48 hrs., 118 r.p.m. → Addn. of substrate (progesterone 100 mg/1 ml acetone)
→ 30°C, 48 hrs., 118 r.p.m. → Sample B and B′

Inoculation (Medium B) → 30°C, 48 hrs., 118 r.p.m. → Add. of substrate (progesterone 100 mg/1 ml acetone)
→ 30°C, 72 hrs., 118 r.p.m. → Sample C and C′

As an example of experiments, results obtained in the case of *A. saitoi* IAM R-1216 are presented in Figs. 4 and 5.

Mass culture of *A. saitoi* IAM R-1216 was carried out, using 4 g of progesterone and 5 L of the medium. It was thereby found that shake culture proceeding for 120 hours after the addition of progesterone effected oxygenation of the progesterone added almost completly. In this case, the degree of oxygenation of progesterone was examined with the elapse of time and it was found that fermentation of 120 hours is required.

D. Discussion

In order to effect the oxygenation of steroids by a fungus, it is imperative that a large amount of fungal cells be produced and the amount of fungal cells produced during 48 hours prior to the addition of a substrate govern the oxygenation of steroids. From such a view, the substitution of one-half of the amount of peptone with ammonium nitrate, thereby increasing the amount of ammonium-nitrogen and nitrate nitrogen,

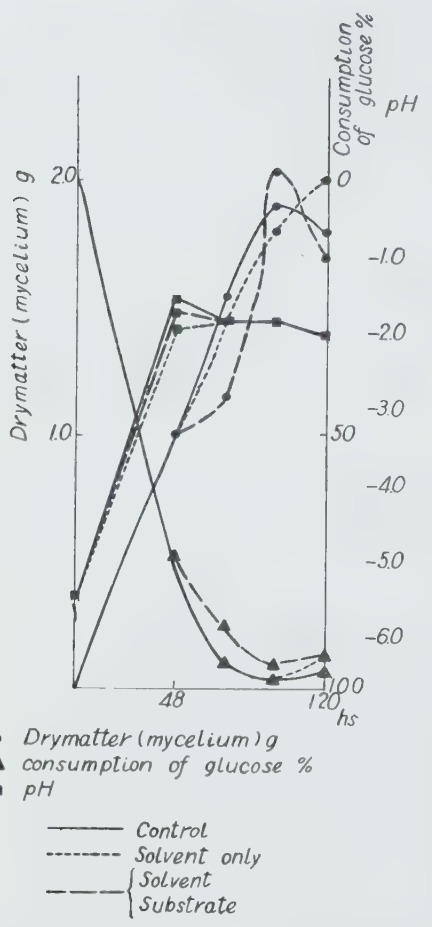


Fig. 4. Variation of pH. consumption of glucose and dry matter of mycelium in the period of fermentation by *A. saitoi* IAM R-1216.

also increasing the ration of nitrogen to carbon was very effective in increasing the amount of fungal cells. The use of propylene glycol as a solvent for the substrate was convenient as the fungus itself, was able to utilize this as its energy source; nevertheless it tended to transfer to the ethyl acetate layer during extraction and to prevent crystallization so that it was considered to be unsuitable as a solvent.

The harmful effect of acetone is apparent with 5 ml of acetone per 100 ml of the medium but as the solubility of steroid contained is very large and a good result can be obtained by the use of about 1 ml per 100 ml of the medium. The oxygenation rate was higher when the substrate was added as a solution in a small amount of methanol or acetone rather than the substrate added alone.

A difference was found in the case of oxygenation between the crystals added after pulverization of the substrate in a mortar and the crystals that precipitated out of the culture liquid after addition as a solution in organic solvent. The latter appeared to be more easily oxygenated by the *Aspergillus* fungi.

Based on the oxygenation of 1 g of steroid added for each L of the medium, the fermentation of 120 hours after addition of the substrate was considered most suitable but, if the amount of substrate is regulated by the strength of a particular strain to oxygenate steroids complete oxygenation can be effected in a shorter period of fermentation.

3. Mass Culture and Oxygenation Product

From the foregoing experimental results, optimal conditions for the formation of 11α-hydroxy- and 6β, 11α-dihydroxyprogesterone from progesterone by partial oxygenation with *Aspergillus* sp. would be as follows:

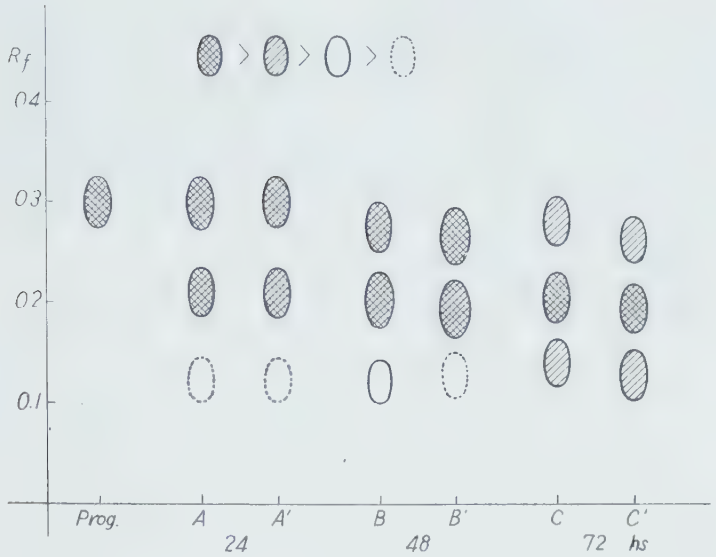


Fig. 5. Paper chromatography in various fermentation time after addition of substrate by *A. saitoi* IAM R-1216.

The fungus is inoculated in medium B, shake cultured (118 r.p.m.) for 48 hours at 30°C, and the substrate (progesterone) is added. The substrate is added as a solution of 100 mg/ml acetone or 100 mg/2 ml methanol per 100 ml of the medium B. Fermentation is then continued under the same conditions for 120 hours.

The rate of various steroids which were crystallized from the culture medium after fermentation should be as follows:

Percentage to recovered total steroid / Strain	Progesterone	11α-OH-	6β, 11α-$(OH)_2$-
A. saitoi IAM R-1216	0	69.5%	30.5%
A. usamii mut. *shirousamii*[2,3] IAM B-407	41.1%	58.9%	0
A. awamori IAM K-0625	54.2%	45.8%	0
A. usamii U.V. mutant IAM 59-1	75.8%	18.3%	5.9%

The ability of *A. saitoi* IAM R-1216 to oxygenated progesterone, to 11α-hydroxy and 6β, 11α-dihydroxy compounds is equal to or better than that of various strains of *Rhizopus*, which had been investigated at the same time in this Institute.[10]

It seemed that the oxygenation products of progesterone produced by the strains of *Aspergillus* are limited to 11α-monohydroxy- and 6β, 11α-

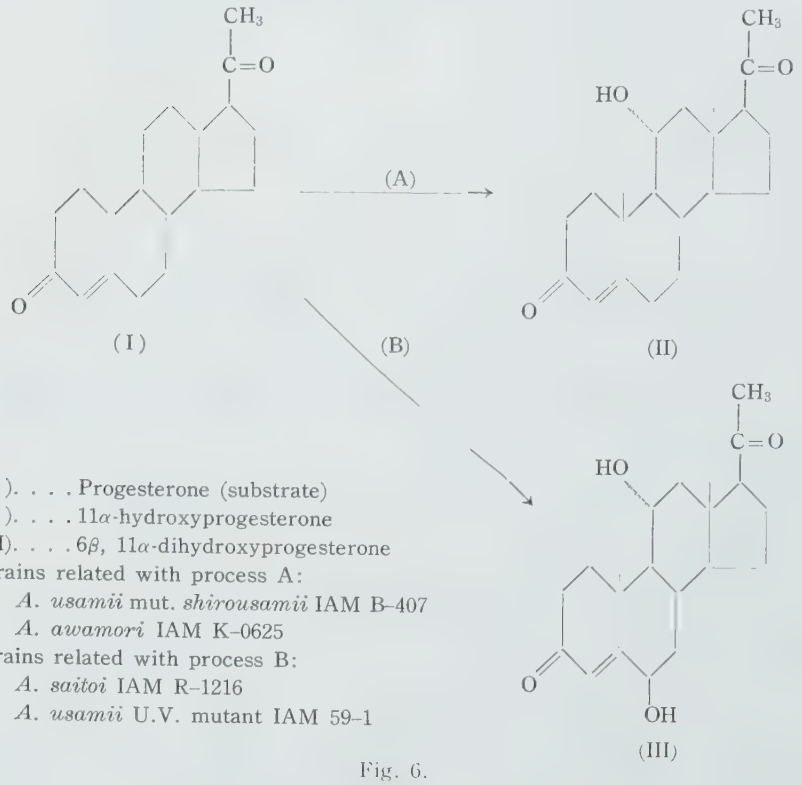
dihydroxyprogesterones. (Fig. 6)

The hydrazone method of Zaffaroni, et al., was adopted for paper chromatography but considering the low Rf values, this point requires further consideration.

As for the results of tank fermentation, an example obtained by the use of a 50 L stainless steel tank with *A. saitoi* IAM R-1216, which had the most active power to oxygenate progesterone, is given here as an example.

Culture conditions:

Medium: 30 L (medium B)
Seeding: 3 L (cultured for 48 hours in a flask)
Aeration: 30 L (equal volume)



(I). . . . Progesterone (substrate)
(II). . . . 11α-hydroxyprogesterone
(III). . . . 6β, 11α-dihydroxyprogesterone
Strains related with process A:
A. usamii mut. *shirousamii* IAM B-407
A. awamori IAM K-0625
Strains related with process B:
A. saitoi IAM R-1216
A. usamii U.V. mutant IAM 59-1

Fig. 6.

Agitation: 250-300/min.
Fermentation:

$$\text{Inoculation} \xrightarrow[30^\circ\text{C}]{24\text{ hrs.}} \text{Progesterone added } \underset{\text{acetone}}{30\text{ g}/500\text{ ml}} \xrightarrow[30^\circ\text{C}]{24\text{ hrs.}}$$

The total procedure required 48 hours and sugar consumption by which the oxygenation was effected almost completely was 96%.

4. Isolation and Identification of Oxygenation Product[9]

Four grams of progesterone was submitted to oxygenation by the foregoing method with *A. saitoi* IAM R-1216, the fungal cells and culture media were extracted with ethyl acetate. The ethyl acetate extract was washed with 2% sodium hydrogen carbonate solution and water, dried over anhydrous sodium sulfate, and the solvent was evaporated under a reduced pressure, affording 6.5 g of a residue. This residue was submitted to chromatography using 150 g of Florisil and the fraction eluted with chloroform and benzene-ethyl acetate mixture (9:1) gave 0.62 g of crystals, which melted at 168–170°C after recrystallization from ethyl acetate. $[\alpha]_D^{20}+169°$ ($c=0.9$, $CHCl_3$). This substance showed no depression of melting point on admixture with 11α-hydroxyprogesterone obtained in another experiment[10].

The fractions obtained by elution with benzene-ethyl acetate (7:3) and (2:1) mixture afforded 0.12 g of crystals which were recrystallized from ethyl acetate to crystals of m.p. 232–234°C, $[\alpha]_D^{20}+152°$ ($c=0.97$, $CHCl_3$).

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found: C, 72.66; H, 8.66.

U.V. λ_{max}^{MeOH}: 236.6 mμ (ε 18000).

I.R.: 3448 (OH), 1698 (20-CO), 1661 (3-CO), 1618 (Δ^4) cm^{-1} (KBr disk).

Acetylation of this substance with acetic anhydride and pyridine at room temperature afforded a diacetate, m.p. 145–148°C, $[\alpha]_D^{20}+66°$ ($c=1.1$, $CHCl_3$).

Anal. Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.51; H, 7.97.

U.V. λ_{max}^{MeOH}: 236.3 mμ (ε 13200).

These data were all in good agreement with those given in previous literature and the substance herein obtained was determined as 6β, 11α-dihydroxyprogesterone.

SUMMARY

1. Comparative experiments were carried out in order for the purpose of examining the ability of 473 strains of *Aspergillus* fungi to form 11α-hydroxy- and 6β, 11α-dihydroxyprogesterone from progesterone. It was thereby found that the strains of black *Aspergillus* and their mutants had stronger oxygenation ability.

2. In order to increase the rate of oxygenation, examinations were made carried out on various cultural conditions and optimal conditions were selected.

3. Mass culture indicated that the fungi could be classified into strains producing only 11α-hydroxyprogesterone (*A. usamii* mut. *shirousamii* IAM B-407, *A. awamori* IAM K-0625) and strains that produced both 11α-hydroxy- and 6β, 11α-dihydroxyprogesterones (*A. saitoi* IAM R-1216, *A. usamii* U.V. mutant IAM 59-1). However, none of these fungi seemed to produce any other products.

4. Tank culture of *A. saitoi* IAM R-1216 was found to effect the complete oxygenation of the substrate in within 48 hours.

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MICROBIOLOGICAL HYDROXYLATION OF STEROIDS III. HYDROXYLATION OF PROGESTERONE BY *SYNCEPHALASTRUM* SP. (1)

TOSHINOBU AŚAI, KYOSUKE TSUDA, KŌ AIDA, EIJI OHKI, TOKUJI TANAKA and MASAKO HATTORI

The Institute of Applied Microbiology, University of Tokyo

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It was found, during the examination of hydroxylation ability of *Rhizopus* sp. described in a previous paper[1], that a strain of *Syncephalastrum* sp., preserved in this Institute, showed strong hydroxylative activity to steroids.

This fungus, when progesterone is used as the substrate, effects hydroxylation in a short period to produce dihydroxyprogesterone, m.p. 222–226°C, and no monohydroxy compound was detected. Application of the fungus for a longer fermentation period of 72 hours after the addition of progesterone effected hydroxylation to trihydroxyprogesterone, m.p. 263–265°C, although its yield was only 5% of the progesterone added. Structural studies on the product will be reported in the following paper[2].

At the same time, some observations were made on the effect of the solvent used in the addition of progesterone, effect of the amount of substrate added, and the course of fermentation, which are described herein.

The authors wish to express their gratitute to Mr. K. Aizawa, Dept. of Agricultural Chemistry, University of Tokyo, for infrared spectral measurements, and to Miss H. Yamanouchi and Mr. K. Satoh of the Microanalysis Division of this Institute for elemental analysis.

EXPERIMENTAL

Examination of Fementation Conditions

The method of incubation was subjected to Peterson[3] followed as below. Shake flasks of 500 ml capacity were cotton-plugged and sterilized and 100 ml of medium, containing 5% glucose, 2% peptone, and 0.3% corn steep liquor, was placed in each flask. The flasks were then sterilized in an autoclave for 15 minutes at 15 lb and the medium showed pH 5.2. Seed from Koji agar slant was inoculated* and submitted to reciprocal shake culture (120

* When growing fungus obtained by shake culture was inoculated, large-sized pellets were formed, growth of mycelium and sugar consumption were poor, and oxygenation activity was decreased.

r.p.m.) for 48 hours in an incubator of 30°C (pH 4.0–4.2) prior to the addition of substrate.

(1) Effect of Solvents Used for the Addition of Substrate: The effect of solvents was investigated with acetone, ethanol, methanol and propylene glycol. These were also used in the study of Mann et al.[4] on biogenesis of hydrocortisone from Compound S with *Cunninghamella blakesleeana*. For 100 ml of the medium, 200 mg of progesterone dissolving in 2 ml of various solvents was added and the effect of solvents on hydroxylation was examined by paper chromatograpy by means of the method of Zaffaroni[5]. The results obtained are listed in Table 1.

Table 1. Effect of Solvents Used for the Addition of Progesterone.

Solvent	pH of medium	Dry wt. of fungus (g)	Residual* progesterone	Hydroxylation product
Acetone	4.2	1.4	##	+
Ethanol	6.0	1.7	#	+
Methanol	6.4	1.7	±	+
Propylene glycol	5.4	1.65	±	+
No solvent	5.4	1.6	##	+

Incubation was conducted for 72 hrs. after addition of progesterone.
* Detected by paper chromatography.

These results indicate that methanol and propylene glycol are suitable as a solvent.

(2) Effect of the Amount of Progesterone Added: Various amounts of progesterone were added to 100 ml of the medium and the products were examined by paper chromatography. Results are shown in Table 2.

It was found that the addition of 100 mg of progesterone was most effective. When 100 mg of progesterone was added as a solution in methanol,

Table 2. Effect of the Amount of Progesterone Added.

Progesterone added (mg)	Methanol	pH	Dry weight of mycelium (g)	State of mycelium	Residual progesterone		Hydroxylation product
					mycelium	broth	
50	2	6.4	2.05	pellet	–	–	+
50	4	4.4	1.05	pulp	–	–	+
100	2	6.4	1.95	pellet	–	–	+
100	4	4.4	1.01	pulp	±	–	+
150	2	6.6	1.80	pellet	+	–	+
150	4	4.6	0.95	pulp	+	–	+
200	2	6.6	2.05	pellet	+	–	+
200	4	4.8	1.00	pulp	+	+	+

oxidation was found to be affected by the volume of the solvent used, as indicated in Table 3.

Table 3. Effect of the Amount of Methanol.

Methanol (ml)	pH	State of mycelium	Dry wt. of mycelium (g)	Residual progesterone		Hydroxylation product
				mycelium	broth	
Nil	5.4	pellet	1.82	++	−	+
1	6.4	〃	1.72	+	−	+
2	6.0	〃	1.72	−	−	+
4	4.2	pulp	0.95	+	−	+
6	4.4	〃	0.85	+	+	+

(3) Course of Fermentation: For 100 ml of the medium, 100 mg of progesterone dissolved in 2 ml of methanol was added, submitted to fermentation, and extraction of the reaction product was conducted every hour. The crude crystals of dihydroxyprogesterone, m.p. 206–214°C, thus obtained, was indicated as the percentage ratio of the progesterone added and shown in Fig. 1. The amount of the oxidation product reached its maximum after 6 hours. After 12 hours, oxidation progressed with the lapse of time and a trihydroxy derivative was formed.

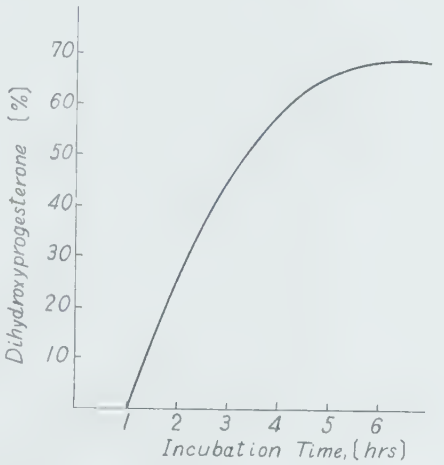

Fig. 1. Periodical Change in Formation of the Product.

(4) Chemical Changes during Fermentation: During the foregoing fermentation, consumption of sugar in the medium, variation of pH, and the increase of mycelium were examined. The results are graphically presented in Fig. 2.

The addition of vegetable oil (sesame oil) and silicone oil (Shin-etsu Silicone Antifoam Agent KS-66, Shin-etsu Chemical Industries, Lt., Tokyo, Japan) as an antifoam agent did not affect oxidation.

Examination of Reaction Products.

The reaction product were examined by mass culture.

(1) Oxidation products after 6 hours' incubation: Ten liters of the medium was prepared and 100 mg of progesterone dissolved in 2 ml of methanol was added for every 100 ml of the medium (total amount of progesterone used, 10 g). After fermentation for 6 hours at 30°C, the mycelium was re-

moved by filtration, and the mycelium and filtrate were each extracted three times with ethyl acetate. The mycelium weighed 110 g on a dried basis. The extract solution was washed twice with 2% sodium bicarbonate solution and water, dried over anhydrous sodium sulfate, and concentrated. The concentrated extract from the mycelium was crystallized from ethanol and after the recrystallization 100 mg of ergosterol, m.p. 145–152°, was obtained. It was confirmed through both ultraviolet and infrared absorption spectra. The mother liquor remaining after removal of the crystals was evaporated and 1.65 g of the residue thus obtained was examined by paper chromatography. The majority was found to be progesterone, no oxidation product being present.

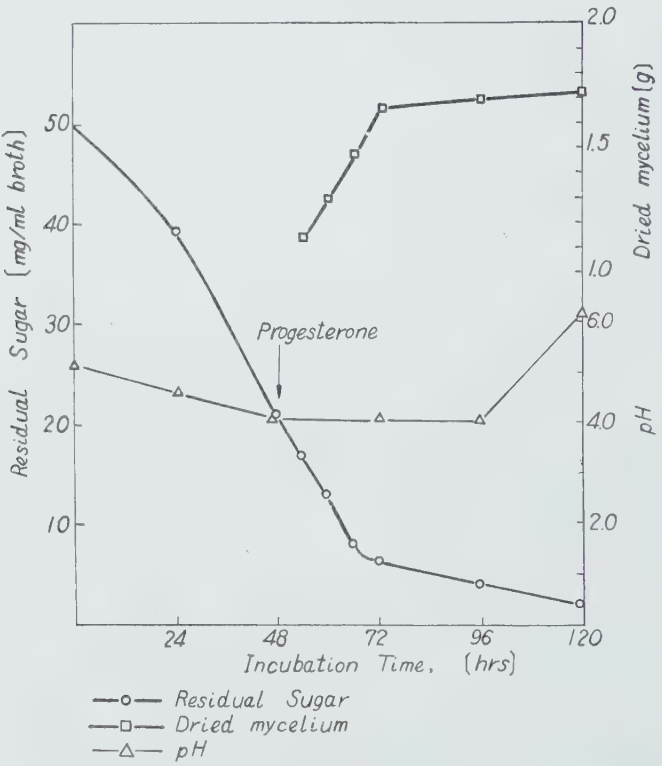

Fig. 2. Chemical Changes during Fermentation.

The crude extract (12.6 g) was obtained from the fermentation liquor, it was recrystalized from ethylacetate, and 6 g of crystals was obtained which melted at 206–214°C. The crystals were treated with chloroform, insoluble matter (0.3 g of m.p. 238–242°C) was filtered off, and the filtrate was evaporated. The residue therefrom was recrystallized twice from ethyl acetate and 2.8 g of crystals, m.p. 222–226°C, thus obtained was identified by elemental analysis as dihydroxyprogesterone.

$[\alpha]_D^{18}+122°$ (c=0.9, $CHCl_3$).

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73.
Found : C, 72.56; H, 8.65.

U.V. λ_{max}^{MeOH}: 240.2 mμ (ε 17000).

I.R. λ_{max}^{nujol}: 3472(OH), 1689(20–CO), 1664(3–CO), 1639(Δ^4) cm^{-1}.

Further concentration of the mother liquor afforded 1.7 g of crystals melting at 218–222°C.

A mixture of 0.45 g of this substance dissolved in 15 ml of pyridine and added with 15 ml of acetic anhydride was allowed to stand over night, poured onto ice water, and extracted with chloroform. The extract was washed consecutively with dilute hydrochloric acid, sodium bicarbonate solution, and water, dried, and the solvent was evaporated. The crude crystals (0.42 g), m.p. 185°C, were purified by column chromatography through Florisil and crystals of m.p. 188–190°C, $[\alpha]_D^{20}+118°$ (c=1.1, $CHCl_3$) were obtained.

Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30.
Found : C, 70.85; H, 8.27.

U.V. λ_{max}^{MeOH}: 237 mμ (ε 15500).

I.R. λ_{max}^{nujol}: 3690(OH), 1739(acetyl CO), 1681(20–CO), 1656(3–CO), 1621(Δ^4) cm^{-1}.

The acetate hereby obtained was found to be a monoacetate.

(2) Oxidation products after 48 hours's incubation: Fermentation was allowed to proceed for 48 hours under condition identical with those in (1); 14.75 g of the extract was obtained from the fermentation liquor. Benzene was added to the residue, the insoluble matter (1.2 g) was collected by filtration, and several recrystallizations from methanol afforded crystals of: m.p. 263–265°C (decomp.), $[\alpha]_D^{20}+170°$ (c=1.1, MeOH). Yield, 0.6 g.

Anal. Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34.
Found : C, 69.59; H, 8.44.

U.V. λ_{max}^{MeOH}: 240.2 mμ (ε 12700).

I.R. λ_{max}^{nuiol}: 3521–3356(OH), 1695(20–CO), 1681(3–CO), 1629(Δ^4) cm^{-1}.

These data confiirmed the product to be trihydroxyprogesterone.

SUMMARY

Syncephalastrum sp. was found to be capable of oxidizing progesterone to form dihydroxyprogesterone requiring a short period of incubation while trihydroxyprogesterone required a long period. These results indicated that steroids are able to be hydroxylated to trihydroxy compounds by one microorganism. The best result in oxidation was obtained by the addition of 100 mg of progesterone to 100 ml of the medium, dissolved in 2 ml of methanol.

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STUDIES ON DEXTRAN FERMENTATION

AKIRA SHINODA

Division of Physiology, The Institute of Applied Microbiology, University of Tokyo

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INTRODUCTION

Dextran which was regarded as a noxious substance to sugar refineries was first separated from sugar syrups as a mucilaginous material in 1870 by Scheibler[1] and genus *Leuconostoc*, dextran forming bacteria, were considered to be harmful. However, since Ingelman and Grönwall[2], Swedish researchers, found it to be very useful to a plasma expander in 1944, many reports on clinical experiments were published in order to confirm its superiority and studies on dextran have made much progress.

Dextran is a polysaccharide of D-glucose polymer in which the glucose units are jointed chiefly through α-1, 6 glucosidic linkages, the others, through α-1, 4 or α-1, 3 glucosidic linkages, and its ratio varies depending on the strains[3].

Among genus *Leuconostoc*, *L. mesenteroides* and *L. dextranicum* produce dextran from sucrose, but some strains of *Streptococcus* are also said to form dextran from sucrose[4].

Systematic studies on genus *Leuconostoc* were carried out by Hucker and Pederson[5], but our knowlege on the chemical structure of dextran produced by genus *Leuconostoc* is due to the works of Hibbert et al., Stacey et al.[7]-[9], and many other investigators[10]-[13].

Conditions for the production of dextran were studied by Tarr and Hibbert[14], who stated that sucrose was the only substrate suitable for dextran formation.

The nutritional requirements of genus *Leuconostoc* for growth and dextran production are reported by many investigators.[15]-[18]

Studies by Hehre[19]-[21] are available in regard to the enzymatic synthesis of dextran and recent reports on the same theme are also published by Koepsell and Tsuchiya, Shiga.[22]-[26]

The present report deals with (a) the isolation of the strains of *L. mesenteroides* which form dextran in a high yield, (b) optimum conditions for the production of dextran using one of these strains, and (c) the studies on native dextran in large scale production.

EXPERIMENTAL

(I) *Isolation of the Microorganisms.*

The composition of the medium used for the isolation of dextran forming strains was as follows:

Sucrose	10.0%
Yeast extract	0.05%
Peptone	0.1%
K_2HPO_4	0.5%
NaCl	0.1%

These ingredients were dissolved in distilled water and pH was adjusted to 7.0–7.2 and sterilized under a pressure of 15 lb. for 20 min..

Raw sugar, vegetables, fruits, canned vegetables and canned fruits were employed as separation sources.

The selection of the useful microorganisms was performed according to the following methods. Samples for isolation were cultured in the medium described above for a period of 24–48 hours at the temperature of 25°C. Two or three loopfuls of the culture thus obtained were inoculated to the liquid medium of the same composition. During these procedures, an increase of viscosity was observed. The microorganism in this medium was plate cultured on koji agar which contained calcium carbonate for the purpose of isolating lactic acid bacteria.

By repeating the plate culture several times, the microorganism was purified. The incubation in the liquid medium was repeated again to examine the increase of viscosity.

From about 200 samples, 5 strains were selected on the basis of their potency of mucilage formation.

(II) *Morphological Studies of the Microorganisms isolated.*

1) Microscopic observation.

The results of the microscopic observations of the five cultures after 24 hours' incubation on koji agar are shown in Table 1.

All strains were Gram positive cocci, usually as diplococci, although short or long chains were formed rarely. Gram-stain was examined by a modifi-

Table 1. Sources and microscopic observations of new isolates.

Strain	Source	Size of majority	Endospore	Motility	Gram-stain
T-4	Tomato	0.9 μ	−	−	+
T-2	Tomato	0.9–1.0 μ	−	−	+
N-4	Carrot	1.0–1.1 μ	−	−	+
C-1	Cabbage	0.9–1.0 μ	−	−	+
H-2	Spinach	1.0–1.1 μ	−	−	+

cation of the Hucker method. Endospores of five days' cultures were tested on bouillon agar by the Möllers method.

2) Cultural characteristics.

(a) Liquid cultures.

(i) Peptone water.

Any changes were not observed except a slight turbidity in the medium after one or two days' incubation at 25°C.

(ii) Yeast water.

Every culture became slightly turbid after one day's cultivation at 25°C.

(iii) Koji water.

Every culture became turbid within one day's incubation at 25°C, and white sediment settled to the bottom of the test tube within one week.

(iv) Sucrose bouillon broth.

Turbidity was observed in all test tubes within 24 hours' incubation at 25°C and they became viscous except strain C-1 and N-4. The increase of viscosity of strain C-1 and N-4 appeared after 2 days' cultivation. Bubbles appeared with gas formation.

(b) Agar slant culture.

(i) Koji agar slant.

Slight, milky white, smooth growth were observed after 2 days' incubation at the temperature of 25°C.

(ii) Sucrose bouillon agar slant.

Opaque, viscous and abundant growth were observed in every test tube after 48 hours' cultivation at 25°C, condensed water became white turbid and coagulated.

(c) Agar stab cultures.

(i) Koji agar.

Slight, white, filiform growth along the line of inoculation developed after 48 hours' incubation at 25°C.

(ii) Sucrose bouillon agar.

Irregular, white, viscous growth developed along the line of inoculation after 2 days' incubation at the same temperature, and the media were split by gas formation.

(d) Cultures on gelatine.

(i) Stab cultures on yeast water gelatine.

After 3 days' cultivation, white growth developed slightly along the line of inoculation in all test tubes at 23°C. No liquefaction of gelatine were seen still after 20 days.

(ii) Stab cultures on sucrose bouillon gelatine.

Smooth, irregular, profuse, white mucid growth developed along the line of inoculation in the case of strains T-2, C-1, H-2 and N-4 after 3 days, but in the strain T-4, uniform, white, sta-

lactitic growth developed. No liquefaction was observed after 20 days' incubation.

(iii) Slant cultures on sucrose bouillon gelatine.

Except strain T–4, abundant, white raised, smooth growth developed after 3 days' culture at the temperature of 23°C, although, on the 6th day, strain N–4 seemed to slip down in condensed water gathering at the bottom of the test tube. In strain T–4, white raised, irregular, rough growth developed.

(III) *Physiological Characteristics.*

The following methods were adopted for the determination of the physiological characteristics of the strains isolated. The results are described in Tables 2, 3 and 4.

(a) Liquefaction of gelatine: The strain under investigation was stabbed on sucrose bouillon gelatine at the temperature of 23°C, and was observed after 20 days' or one month's incubation. (Table 2) No liquefaction occurred.

(b) Catalase reaction: One-loopful of the microorganism under investigation was suspended into 10 ml of 3% hydrogen peroxide solution. Catalase reaction was negative in all strains. (Table 2)

(c) Hydrogen sulfide formation: The strain was stabbed in nutrient agar containing 0.05–0.1% of lead acetate. Observation was performed whether or not the color of the medium along the line of inoculation changed to black after one week. The results show that the formation of hydrogen sulfide can not be observed in all microorganisms. (Table 2).

(d) Indole formation: The microorganism under investigation was inoculated in 2% of peptone water and tested by the Ehrich-Böhme method after one week. (Table 2)

(e) Reduction of nitrate: The strain was cultured from 5 or 10 days in peptone water containing 0.1% of KNO_3 (1% of glucose was also added) and this culture was tested on nitrite production from nitrate using α-naphtylamine and sulfanilic acid. (Table 2).

(f) Change in litmus milk: The strain under investigation was inoculated at 25°C for one week. Strain N–4, C–1 showed slight acidic, but other strains showed no change. (Table 2)

(g) Temperature for growth: The strain was cultured for 3 days in a medium of peptone water containing 2% of glucose, and the culture was maintained the following temperature at 15–18°C, 20°C, 25–27°C, 30°C, 35°C and 37°C respectively. Acid produced was titrated with N/10 NaOH every day, and the optimum temperature for growth was determined with the volume of alkali consumed. (Table 2)

(h) Thermal death point: An inoculated koji water medium was heated for ten min. in a water bath at constant temperature and cultured for 2 days at 25°C. Thus, the thermal death point was determined. (Table 2)

(i) Acid production from various carbohydrates: (1) one ml of 1.6% alcoholic solution of B.C.P., per litre of peptone water, was added as the

Table 2. Physiological characteristics of new isolates.

Strain	T-4	T-2	N-4	C-1	H-2
Liquefaction of gelatine	–	–	–	–	–
Catalase reaction	–	–	–	–	–
Hydrogen sulfide formation	–	–	–	–	–
Indole formation	–	–	–	–	–
Reduction of nitrate	–	–	–	–	–
Change in litmus milk	–	–	slightly acidic	slightly acidic	–
Optimum temperature for growth	20–25°C	20–25°C	20–25°C	20–25°C	20–25°C
Thermal death point	90°C (10 min)	80°C (10 min)	80°C (10 min)	90°C (10 min)	80°C (10 min)
Gas production	+	+	+	+	+
Relation to free oxygen	facultative anaerobe	facultative anaerobe	facultative anaerobe	facultative anaerobe	facultative anaerobe

indicator and the pH was adjusted to 7.0 and divided by 5 ml into test tubes (2) 4.0% solutions of various carbohydrates dissolved in distilled water were prepared. After all solutions were sterilized for 20 min. at a pressure of 15 lb., 5.0 ml of solution (1) was added to 5 ml of solutions (2) under aseptic conditions. The strain under investigation was inoculated in these media and incubated at the temperature of 25°C for 10 days. The acid produced was titrated with N/10 NaOH until original color of the indicator recovered. The results which are listed in Table 3, show that all strains produce large amounts of acid from sucrose, fructose, maltose, glucose and pentoses (arabinose and xylose).

(j) Relation to free oxygen: All strains grew well throughout sucrose bouillon deep agar medium in test tubes and were considered as facultative anaerobes. (Table 2)

(k) Products obtained from glucose by fermentation: 400 ml of peptone water medium containing 3% of glucose and 1.5% of calcium carbonate was prepared in a 500 ml-Erlenmeyer flask and sterilized under a pressure of 15 lb. for 20 min., and 10 ml inoculum of 24 hours' culture of the same composition was added and it was held at 25°C for 14 days. Qualitative and quantitative determinations were carried out with this culture according to the following procedures.

The fermented broth was filtered to remove the remaining calcium carbonate and bacterial cells, making alkaline, and then steam distilled. This distillate was treated for the determination of ethanol by Davy's method and Agulhon's method. The remainder of the distillation was made acidic with sulfuric acid and was steam distilled again. This distillate was used for the determination of volatile acids.

Table 3. Amounts of N/10 NaOH required to neutralize the acid produced in 10 ml of 2% solution of various carbohydrates.

Carbohydrates	Volume (ml) of N/10 NaOH required to neutralize the acid				
	T-4	T-2	N-4	C-1	H-2
Xylose	1.1	0.8	1.1	0.7	1.2
Arabinose	1.2	1.7	0.9	1.0	1.6
Glucose	0.8	0.5	0.7	0.5	0.5
Fructose	2.2	1.9	0.8	1.0	1.9
Mannose	0.8	0.8	0.6	0.7	0.8
Galactose	0.3	0.3	0.44	0.46	0.35
Maltose	0.85	0.85	0.4	0.55	0.8
Sucrose	1.75	1.7	1.5	1.0	1.9
Lactose	0.0	0.0	0.4	0.4	0.0
Raffinose	0.12	0.05	0.2	0.3	0.0
Dextrin	0.12	0.2	0.1	0.1	0.08
Inulin	0.0	0.0	0.0	0.0	0.0
Starch	0.0	0.0	0.0	0.0	0.0
Ethylen glycol	0.0	0.0	0.0	0.0	0.0
Erythritol	0.0	0.0	0.1	0.0	0.0
Mannitol	0.3	0.3	0.25	0.2	0.3

The remainder of the second distillation was extracted with ether for 48 hours, and the ether-extracted fraction was neutralized with barium hydroxide and dried up. The barium salt thus obtained was dissolved by adding 80% ethanol. Lactic acid was detected by the Fletcher-Hopkins reaction and estimated on the determination of barium.

Extraction with ether was carried out again with the ethanol solution of barium lactate by acidifying with sulfuric acid, and the ether soluble substance was dissolved in water after the removal of ether, decolorized by active carbon, neutralized with zinc carbonate to form zinc lactate. The white crystal of zinc lactate was dried and weighed. After heating up to 110°C,

Table 4. Products obtained from 3% of glucose medium fermentation.

Product \ Strain	T-4	T-2	N-4	C-1	H-2
*Lactic acid (%)	38.2	37.5	39.6	39.6	41.2
*Acetic acid (%)	7.5	6.6	5.3	7.1	7.2
Ethanol	+	+	+	+	+
CO_2	+	+	+	+	+
Optical form of lactic acid	D	D	D	D	D

* The percentage of these acids based on the amount of glucose fermented.

the substance was reweighed for the determination of crystal water (12.89%).

Rotation of the crystal was determined with a polarimeter ($[\alpha]_D$, +7.7°).

It was confirmed that all strains produced laevorotary lactic acid. The results are presented in Table 4.

From the above described properties, they were identified to typical strains of *Leuconostoc mesenteroides* by Bergey's Manual of Determinative Bacteriology, 6th edition (1948).

(IV) *The Mucilage Production from Sucrose.*

Viscosity of the medium used in experiment (1) increased extremely after 4 days' incubation at 25°C; an equal volume of methanol was then added to the broth. A white gummy material was precipitated.

The supernatant was decanted and the gummy mass dissolved again in water and reprecipitated with methanol. After this procedure was repeated again, the precipitate was washed with methanol and dried at 105°C. Substances thus obtained did not reduce Fehling's solution and contained amounts less than 0.5% of ash and 0.1% of nitrogen.

The hydrolysates of 1% dextran solution contained 94–96% of reducing sugar as glucose which were decomposed for 6 hours with N-HCl. Each osazone formed as a yellow needle crystal with phenylhydrazine hydrochloride from these hydrolysates was identified as glucosazone by its crystal form and melting point. (m.p. 208°C). Mannose did not exist because white phenylhydrazone precipitates were not observed. Fructose was also not detected in these hydrolysates by Roe's method[27]. When each solution was treated with either thiobarbituric acid or phloroglucinol, pentose and uronic acid reactions were negative.

Only glucose was detected by paper chromatography with the hydrolysates.

These results mentioned above indicated that these gummy materials were solely constituted from glucose. In addition to this they were dextrorotating, $[\alpha]_D$, +195–205° (in a solution of N-NaOH). Therefore, these mucilages were confirmed to be dextran.

(V) *Optimum Conditions for Dextran Production by Leuconostoc mesenteroides.*

To determine the most satisfactory strain among new isolates, the following examinations were carried out: (1) dextran producing ability from sucrose. (2)* solubility of the dextran produced. (3)** the ratio of α-1, 6

* Dextran precipitated with methanol is contained considerable amounts of impurities derived from the medium, so purification is performed by dissolving in water and reprecipitating with methanol. For this reason, native dextran is necessary to be soluble in water.

** It is stated that satisfactory dextran for a plasma expander requires a high proportion of 1, 6 linkages.

Table 5. Properties of dextran produced by five strains.

Strain	Dextran producing ability	Solubility of dextran in water	Ratio of 1, 6-linked units[28] per non 1, 6-linked units
T-4	##	less soluble	10.0
T-2	##	less soluble	5.7
N-4	##	soluble	10.0
C-1	##	soluble	4.9
H-2	##	less soluble	14.0

glucosidic linkages in dextran produced.

From the results of these tests as shown in Table 5, strain N-4 was found to be the most satisfactory strain for the production of dextran.

Succeeding studies on optimum conditions of dextran production were performed with this strain.

(a) Optimum pH for the production of dextran from sucrose.

A basal medium containing the following composition was prepared: sucrose, 10%; peptone, 0.1%; K_2HPO_4, 0.5% and NaCl, 0.1%; dissolved in distilled water.

Fifty ml of this solution was brought up to various pH values with N-NaOH, N-HCl under aseptic conditions in 100 ml of tared Erlenmeyer flasks. Contamination tests were carried out by keeping them at 37°C for 2 days. Measurements of pH were made with a glass electrode. Each solution was inoculated with two loopfuls of a 24 hours'-old liquid culture (the same composition as with pH 7.0) and all of them were incubated for 5 days at 25°C.

The dextran was precipitated with an equal volume of methanol after incubation, the supernatant decanted from dextran after standing for 24 hours, and dried at 105°C, then kept in a vacuum desiccator over anhydrous calcium chloride at room temperature till the weight became constant.

The yields of dextran production were calculated from the following relationship and expressed as the percentage of theoretical yield.

$$n\text{-Sucrose} \longrightarrow \underset{\text{Dextran}}{(C_6H_{10}O_5)_n} + n\text{-}\underset{\text{Fructose}}{C_6H_{12}O_6}$$

Table 6. Effect of various pH on dextran yields.

pH of medium	Dextran yield (%)	pH of medium	Dextran yield (%)
5.4	16.8	7.4	88.2
5.8	42.6	7.6	67.2
6.0	50.5	8.0	58.8
6.2	63.0	8.2	58.8
6.4	67.2	8.4	58.8
6.8	88.2	8.8	58.8
7.0	90.4	9.0	16.8
7.2	90.4		

The results of the experiment, as shown in Table 6, indicate that dextran yield is maximum in the range of pH 6.8 and 7.4.

(b) Optimum concentration of peptone.

Peptone was added to solutions containing 10% sucrose, 0.5% K_2HPO_4 and 0.1% NaCl, to make final concentration up to 5%, 2.5%, 1%, 0.5%, 0.1%, 0.05%, 0.01% and 0.0%, respectively, and all solutions were brought up to pH to 7±0.2. In succeeding experiment, the pH of the media were brought to the same values and subsequent procedures were the same as in experiment (a).

Peptone concentration of 0.05–0.1% were sufficient for the production of dextran as shown in Table 7. Almost the same yields were even obtained when peptone concentration exceeded 0.1%, although a decrease in yields were reported by Tarr and Hibbert.

Table 7. Influence of various concentrations of peptone on dextran production.

Peptone concentration (%)	Dextran yield (%)	Peptone concentration (%)	Dextran yield (%)
0.0	33.6	0.5	88.2
0.01	75.6	1.0	90.4
0.05	88.2	2.0	88.2
0.1	88.2	5.0	88.2

(c) Optimum concentration of sucrose.

Aqueous solutions containing 0.5% K_2HPO_4, 0.1% NaCl and 0.1% peptone and various concentrations of sucrose were prepared (sucrose concentrations 30%, 20%, 15%, 10%, 5%, 2%).

The results, recorded in Table 8, show that a high dextran yield is obtained in low sucrose concentrations and when the concentration exceed 15%, the yield decreases. About 10% of sucrose concentration is seemed to be suitable for the production.

Table 8. Effect of various concentrations of sucrose on dextran yields.

Sucrose concentration (%)	Dextran yield (%)
2	94.5
5	96.8
10	90.4
15	87.0
20	60.8
30	20.9

(d) Optimum concentration of K_2HPO_4.

Aqueous solutions containing 10% sucrose, 0.1% NaCl and 0.1% peptone were prepared and K_2HPO_4 was added to these media so as to make final

concentration up to 1%, 0.5%, 0.1%, 0.05%, 0.01% and 0.0%, respectively.

As shown in Table 9, the optimum concentration of K_2HPO_4 for production was in the range 0.1–0.5%.

Table 9. Effect of various concentration of K_2HPO_4 on dextran yields.

K_2HPO_4 concentration (%)	Dextran yield (%)
0.0	16.8
0.01	58.8
0.05	73.5
0.1	90.4
0.5	90.4
1.0	25.1

(e) Optimum concentration of NaCl.

Solutions containing 10% sucrose, 0.5% K_2HPO_4 and 0.1% peptone were prepared and various amounts of NaCl were added to these media to form final concentrations of 1%, 0.1%, 0.05%, 0.01% and 0.0%, respectively.

The optimum concentration of NaCl was found to lie between 0.1–1.0% for dextran production and dextran yield seemed to decrease at the concentrations of less than 0.05% NaCl, as shown in Table 10.

Table 10. Influence of various concentration of NaCl on dextran yields.

NaCl concentration (%)	Dextran yield (%)
0.0	63.0
0.01	82.0
0.05	82.0
0.1	88.2
0.5	88.2
1.0	86.2

(f) Effect of yeast extract for the production of dextran.

Yeast extract was reported to be a growth promoting substance for genus *Leuconostoc* according to Hucker and Pederson.

Hassid and Barker[29], Jeanes et al.[30] used yeast extract as an ingredient of media for dextran production.

Solutions containing 10% sucrose, 0.1% peptone, 0.1% NaCl, 0.5% K_2HPO_4 and various concentrations of yeast extract were prepared and the pH was adjusted to 7 ± 0.2 and sterilized.

The same procedures as those above mentioned were performed at 24, 48 and 120 hours' incubation.

In this experiment, 0.5% of K_2HPO_4 was suitable because the pH of media lowered rapidly caused by the existence of yeast extract.

As shown in Table 11, the addition of yeast extract proved to be very satisfactory, and in the media containing yeast extract with a concentration

Table 11. Effect of yeast extract on dextran production.

Concentration of yeast extract in solution (%)	Yield of dextran (%)		
	24 hours	48 hours	120 hours
0.0	18.9	50.5	88.2
0.01	50.5	92.5	90.4
0.02	50.5	92.5	92.5
0.05	90.4	94.5	94.5
0.1	88.2	94.5	92.5
0.2	88.2	94.5	92.5

higher than 0.05%, dextran was produced almost theoretically within 24 or 48 hours' incubation. Dextran yields did not decrease even in lower concentrations after 48 hours' cultivation.

(g) Shaking culture.

Amounts of 100 ml of solutions which contained 10% sucrose, 0.1% peptone, 0.05% yeast extract, 0.5% K_2HPO_4 and 0.1% NaCl were prepared in 500 ml shaking flasks and the pH was adjusted to 7±0.2 and sterilized. After inoculating, the flasks were incubated at 25°C on a reciprocating shaker at the rate of 110 r.p.m. with a stroke of 8 cm.

Table 12 gives a comparison of dextran yields between shaking and stationary culture at 24 and 36 hours' incubation. The yield of dextran was

Table 12. The comparison of dextran production between shaking culture and stationary culture.

	Yield of dextran (%)	
	24 hours	36 hours
Shaking culture	23.05	48.4
Stationary culture	88.2	92.5

less in the shaking culture and no increase of viscosity was observed. However, both growth and increase of viscosity at the initial stage of 6–12 hours' incubation were observed to be in a high extent in case of shaking culture.

(VI) *Trial Test on the Dextran Production by Tank.*

Dextran fermentation was carried out in a 200 L stainless steel tank with strain N-4, in accordance with the results of experiment (V).

An aqueous solution containing sucrose 10%, yeast ext. 0.05%, peptone

0.1%, K_2HPO_4 0.5%, NaCl 0.1% was used as a medium and the pH was adjusted to 7.0±0.2 and sterilized under a pressure of 15 lb. for 20 min..

(a) Method of analysis.

(i) Viscosity: Viscosity measurements were made at 25°±0.05°C with a Ostwald viscosimeter.

(ii) pH: pH measurements were made with a glass electrode.

(iii) Total acidity: 10 ml of the broth was titrated with N/10 NaOH using B.T.B. as an indicator. Total acidity was calculated with the volume of alkali consumed.

(iv) Reducing sugars: Reducing sugars were analysed by the Fehling-Lehmann-Schoorl method and calculated as fructose.

(v) Dextran produced: Dextran was assayed by the method described in experiment (V).

(vi) Optical rotation: Optical rotations were read with the light from a sodium vapor lamp.

(vii) Nitrogen: Nitrogen analysis were made by the micro-Kjieldahl procedure.

(b) Preparation of inoculum.

One-loopful of the microorganism was inoculated to 100 ml of the medium in a test tube and incubated for 24 hours at 25°C.

This culture was transferred to 200 ml of the medium in a 300 ml Erlenmeyer flask. After a 24 hours' incubation at 25°C, this was transferred to a 5 L-flask containing 4 L of the medium, incubated for 24 hours at 25°C and used as inoculum.

(c) Chemical changes in the medium during fermentation.

One hundred L of the medium was prepared in a 200 L stainless steel fermentor and incubated at 25°C after inoculation. The medium was agitated at a rate of 80 r.p.m.

Withdrawal of test samples were performed periodically under aseptic conditions. Viscosity, pH, total acidity, reducing sugars and dextran production in the culture medium were measured with the methods previously described.

The results are shown in Fig. 1.

The viscosity of the broth increased rapidly after 12 hours' cultivation and showed a maximum value at about 24 hours, thereafter remarkable decrease was observed. Decrease of viscosity slowed down after 48 hours' cultivation and a slight decline still continued after 7 days' incubation. This seemed to show that the dextran of high molecule once produced was degraded to a lower molecule as will be discussed later.

The pH dropped as the fermentation proceeded and reached approximately 4.4 after 24 hours, thereafter it lowered slowly up to 48 hours, after which a steady value of 3.7 was maintained.

Total acidity increased rapidly for the first 48 hours' cultivation and a slight increase was to be seen after the following incubation.

Reducing sugars which were produced in proportion to the dextran formation, showed a maximum value during 24–30 hours, then a slight decrease was observed after extended incubation. This fact suggested that fructose produced during the fermentation was considered to be metabolized.

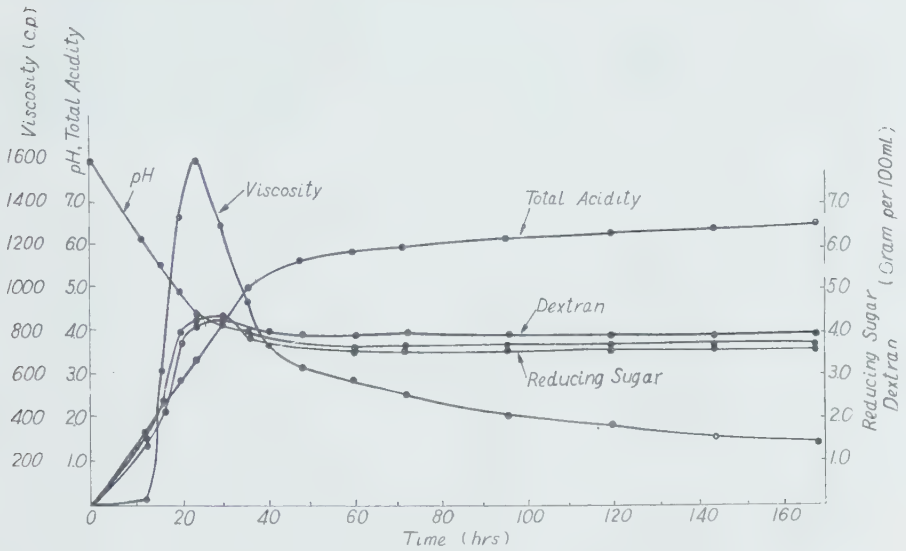

Fig. 1. Chemical changes in the culture medium.

Dextran production reached its maximum after 24–30 hours, then a slight decrease was observed after prolonged incubation.

This fact showed that the amount of native dextran precipitated at a 50 volume % methanol concentration decreased. The supernatant of 24 hours' culture broth from which native dextran was separated by the 50 volume % methanol concentration no longer precipitated with further addition of methanol. On the contrary, the supernatant of the prolonged culture of more than 48 hours became white turbid by the addition of methanol and precipitation was observed.

(d) Fractional precipitation of native dextran with methanol.

Analytical data of native dextran are shown in Table 13. They were obtained from the broth of 24 hours' culture, (preparation A) and 7 days' culture (preparation B) by the addition of an equal volume of methanol and purified by reprecipitation with methanol as stated in experiment (IV).

Methanol was added to 2% aqueous solutions of these preparations in order to make various concentrations and these samples were kept at 25°C for 5 hours. The dextran fractions thus obtained, at variout methanol concentrations were dried and weighed. Results are shown in Fig. 2.

Precipitation in preparation A was almost completed at about 38 volume

Table 13. Data on native dextran from each preparation

Dextran	Yield from sucrose (%)	N (%)	Ash (%)	$[\alpha]_D^{25°}$ (in N NaOH)	$[\eta]$*
Preparation A	42.5	0.07	0.05	198°	1.1
Preparation B	40.0	0.06	0.04	197°	0.95

* Intrinsic viscosity $[\eta]$ were measured as follows: Relative viscosity of about 1%, 0.5% and 0.1% aqueous solutions were measured and plotted against concentration, giving a line which could be extrapolated to the viscosity axis, the intercept being $[\eta]$.

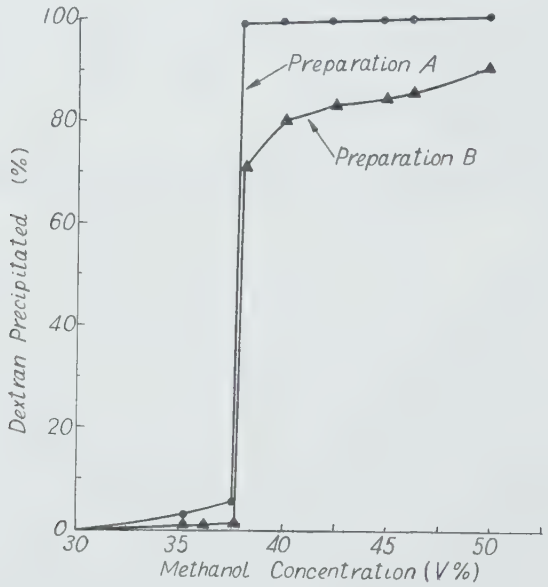

Fig. 2. Precipitability of native dextran from each preparation with methanol.

% methanol concentration. On the other hand, in preparation B, only 71% of dextran was precipitated at the same concentration and precipitation continued slowly by further addition of methanol.

Even at 50% methanol concentration, approximately 90% of dextran was precipitated and its supernatant showed white turbidity.

This fact seemed to show the native dextran once produced degraded during extended incubation to a lower molecule.

DISCUSSION

As a plasma expander, the type of glucosidic linkages in dextran is very important. Straight chains of glucose are jointed through α-1, 6 glucosidic

linkages in dextran and branchings occur at α-1, 3 or α-1, 4 linkages on the contrary to glycogen or starch. α-1, 6 glucosidic linkages are attacked slowly by tissue enzymes, but other linkages are decomposed more rapidly. This has a clinical significance, because it is useless for a plasma expander, to be easily decomposed and eliminated rapidly from the blood stream.

The selection of the strains which produce dextran having a high proportion of α-1, 6 linkages has become a procedure of much importance from this point of view. The solubility of dextran in water is also important, because purification of native dextran is carried out by dissolution in water and then by precipitation with organic solvents such as methanol, ethanol or acetone.

Leuconostoc mesenteroides N–4, a new isolate of the author is satisfactory from this point as a strain NRRL B–512 which is widely utilized for the commercial production of dextran in the United States. For the dextran formation with this strain, the medium having the following composition is seemed to be most suitable: sucrose 10%, yeast extract 0.05%, peptone 0.1%, NaCl 0.1%, K_2HPO_4 0.5% and pH to 7 ± 0.2.

Culture by shaking was not so satisfactory for production, as in stationary culture. Dextran forming enzyme, as reported by Hehre[20] does not seem to be dependent on oxidative processes.

Dextran formation completed within about 24–30 hours' incubation by large scale fermentation and the yields of dextran from sucrose reached as much as 42.5%, that being approximately 90% of theoretical value. The decrease of viscosity in the culture medium after extended cultivation appears to be caused by the degradation of the dextran.

The dextran from 7 days' culture was observed to be degraded when compared with 24 hours' culture. Jeanes[30] and Hamdy[31] suspected that this was caused by the dextran degrading enzyme in the culture medium which was formed by autolysis. But as further investigations are considerded necessary on this point, the results of the experiments concerning this problem will be reported in the near future.

SUMMARY

(1) For the purpose to obtain microorganisms having high dextran producing ability, five strains were isolated from various sources.

These were lactic acid bacteria which were able to produce slime from sucrose.

(2) Morphological and physiological investigations of these strains were carried out and a gummy material obtained from sucrose was identified as dextran. These strains were decided to be assigned to *Leuconostoc mesenteroides*.

(3) Dextran production by these strains was examined and it was confirmed that strain N–4 was the most satisfactory strain.

Optimum conditions for dextran formation with this strain are as follows:

(a) Optimum pH ranged between 6.8 and 7.4.

(b) Peptone concentrations of 0.05–0.1% were optimum for the production of dextran.

(c) A high yield of dextran production was obtained in low sucrose concentrations, and this decreased in a concentration of more than 15% sucrose. About 10% sucrose concentration was considerded to be suitable for dextran production.

(d) K_2HPO_4 0.1% and NaCl 0.1% were found to be optimum for dextran production.

(e) The use of yeast extract was very effective and fermentation was completed within 24–48 hours using a 0.05% concentration.

(f) The production of dextran by shaking culture was found to be inferior to stationary culture.

(4) Dextran fermentation was carried out in a large scale using a 200 L fermentor.

(a) The viscosity of the broth reached a maximum after 24 hours then decreased rapidly.

(b) pH showed 4.4 after 24 hours then lowered slowly to 3.7 and maintained this value throughout the fermentation.

(c) Total acid increased rapidly up to 48 hours, and then a slight increase was observed.

(d) Dextran formation reached a maximum during 24–30 hours' incubation and the yield showing about 42.5% of sucrose.

(e) Reducing sugars were produced in proportion to dextran formation, and a slight decrease was observed after it reached a maximum during 24–30 hours.

(f) Fractional precipitation of native dextran which were obtained from both the 24 hours' and 7 days' culture broth respectively, was performed and it was found that the molecule of native dextran separated from the latter was lower than that of the former, so dextran once produced seemed to degraded by prolonged incubation.

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SYNCHRONOUS CULTURE OF *CHLORELLA* WITH SPECIAL REFERENCE TO THE PROCESSES OF ASSIMILATION OF POTASSIUM, MAGNESIUM AND IRON

TAMOTSU KANAZAWA

The Tokugawa Institute for Biological Research, Tokyo

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INTRODUCTION

The study of physiological and biochemical processes occurring in the life cycle of *Chlorella* has been greatly facilitated since the technique of synchronous culture of the alga was developed in our laboratory (1–4). By elaboration of the technique we are now able to analyse the life cycle of algal cells into the following seven successive stages (see Fig. 1).

(a) "Nascent dark cells" (D_n), which are the young cells newly produced from mother cells.

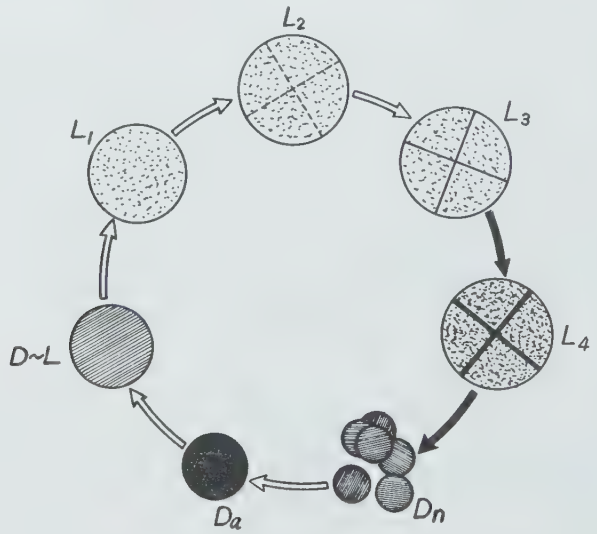

Fig. 1. Schematic representation of the life cycle of *Chlorella*. White arrows indicate the light processes, while black arrows show the processes that can occur independently of light. Sizes of circles correspond approximately to the relative sizes of cells and the extent of darkening indicates the relative photosynthetic activity.

(b) "Active dark cells" (D_a), the most chlorophyll-rich and photosynthetically active cells which are derived from D_n when illuminated.

(c) Cells of transient stage between dark and "light" cells ($D \sim L$).

(d) "Immature light cells" (L_1), which are large in size, but not yet ripe enough to perform cell division when incubated in the dark.

(e) "Half-mature light cells" (L_2), which can only partially divide when kept in the dark.

(f) "Mature light cells" (L_3), which can completely divide when kept in the dark.

(g) "Fully ripened light cells" (L_4), which are at the stage immediately prior to the process of cell division.

It was observed (3, 4) that the cells at these different stages show more or less different contents in nitrogen, phosphorus, sulphur as well as in crude proteins, lipids, carbohydrates, nucleic acids, chlorophyll, etc. The present experiments were performed with the view to examine whether and in what manner the contents of algal cells in potassium, magnesium and iron will change during the course of their life cycle.

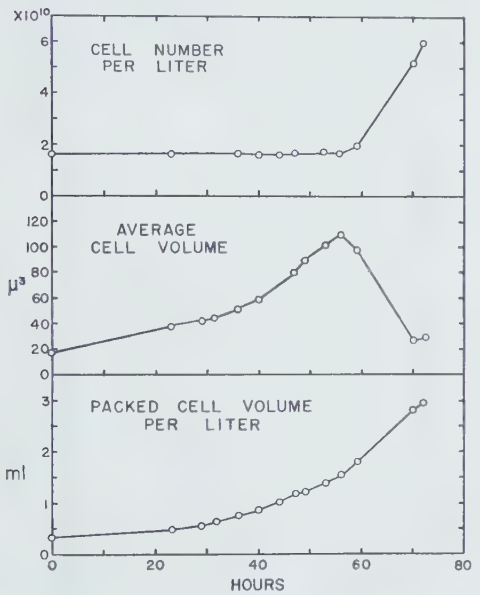

Fig. 2. An example of synchronous culture of *Chlorella* starting from "active dark cells". Illumination: 10 kilolux. Temperature: 15°–16°.

METHODS

The experimental organism was *Chlorella ellipsoidea*, and the culture methods employed were virtually the same as those reported previously (3, 4). The preultures were run at 20°–25° under illumination with daylight fluorescent lamps of 8–10 kilolux in intensity, and the subsequent synchronous culture starting from "active dark cells" was conducted at 15°–16° under illumination with a series of daylight fluorescent lamps, about 10 kilolux in intensity at the surface of the culture. The culture medium used was of the following composition: per liter, KNO_3 5.0 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, KH_2PO_4 1.25 g, $FeSO_4 \cdot 7H_2O$ 0.003 g, Arnon's "A5" solution 1 ml (5). During the experiment the cultures were constantly aerated with air containing 5 percent CO_2. The process of cell development was followed by measuring: (i) the number of cells per liter (using a hemacytometer), (ii)

the statistical distribution of cell size (by means of an ocular micrometer), and, (iii) the packed volume of cells per liter (using a hematocrit). An example of the data of synchronous culture is presented in Fig. 2. In this case the cell division began at the 59th hour of the experiment and was completed within about 12 hours.

At various stages of synchronous culture, certain portions of cell sample were taken out, and after washing three times with distilled water, they were analysed for their dry weight, ash weight and cation contents using the following methods. The cells (about 100 mg in dry weight) were dried to constant weight using infra-red lamps, and after being charred in an oven, kept in a muffle furnace at 450°–480° for 24 hours. When the ashing was incomplete, the sample was dissolved in a minimum quantity of concentrated nitric acid and heated again in the furnace. After completion of the ashing, the sample was dissolved in 1 ml of concentrated hydrochloric acid, and passed through a column of Amberlite IRA 400 (3 ml in volume) with subsequent scrubbing using 20 ml of distilled water (at the velocity of 0.5 ml per minute). The solution obtained was made up to 25 ml in a volume flask, and subjected to the determination of K, Mg and Fe. Potassium and magnesium were determined by means of flame photometry (at 404.6 mμ for K and 371 mμ for Mg) using the Beckman DU spectrophotometer. Determination of iron was conducted by application of the orthophenanthroline method (6).

RESULTS

Change in content of total ash. The total ash content of algal cells showed a characteristic change during the course of the life cycle. The data obtained at successive stages of cell development are presented in Fig. 3, in which the content is given both on the per cell basis and in percent of dry weight. For comparison, the change of dry weight of single cells is also shown in the figure. As may be seen, the ash content per cell remained almost unchanged at the stages from D_n to L_1, and increased markedly at later stages (L_2, L_3 and L_4). Since the weight of single cells increased appreciably during the period from D_n to L_1, the ash content in percent of cell substance showed a marked decrease during this period. The percentage

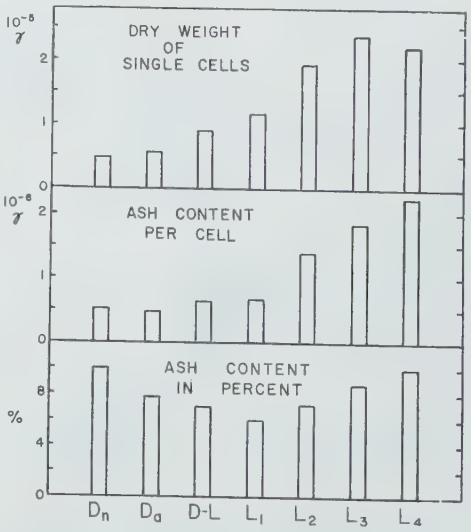

Fig. 3. Change of total ash content during the course of life cycle, as compared with the change of dry weight of single cells.

was lowest (6.5 percent) at L_1 and highest (10 percent) at D_n and L_4.

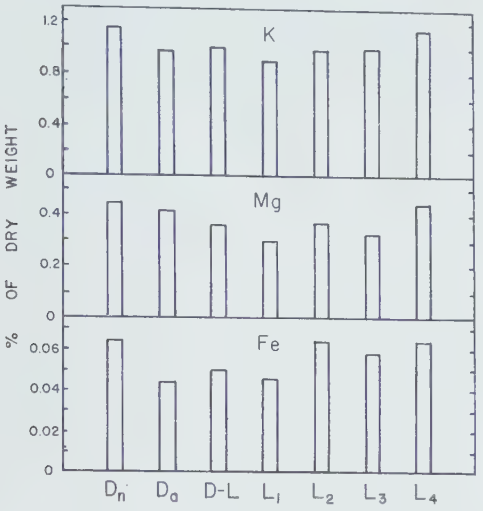

Fig. 4. Percentage (on a dry weight basis) of potassium, magnesium and iron as found in cells of different developmental stages.

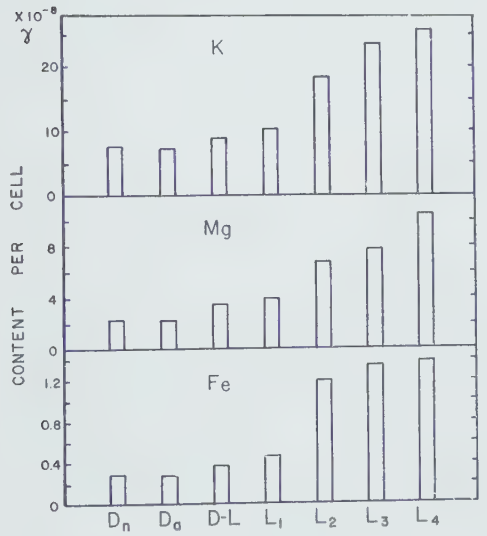

Fig. 5. Changes of contents (per cell) of potassium, magnesium and iron during the course of life cycle.

Changes in contents of potassium, magnesium and iron. Changes in contents of potassium, magnesium and iron are shown in Figs. 4 and 5, the contents being given in percent of dry weight and on the per cell basis, respectively. In terms of percentage of dry weight, the content of potassium remained relatively constant throughout the life cycle, indicating that the assimilation of potassium occurred almost keeping pace with the increase of total cell mass. On the other hand, the contents (in percent) of magnesium and iron decreased markedly during the stages from D_n to L_1, and increased sooner or later during the later stages.

DISCUSSION

We have seen that potassium was assimilated by algal cells almost keeping pace with the photosynthetic assimilation of the bulk of cell material, whereas magnesium and iron were assimilated more in the ripening stage (the stage of light cells) than in the growing stage (the stages from D_n to L_1). The role of potassium in the physiology of algal cells is at present still quite obscure. It may only be noticed here that the occurrence of process similar to that observed for the assimilation of potassium has thus far been observed also in the assimilation of nitrogen and in the formation of bulk protein and ribonucleic acid in algal cells (3, 4).

On the other hand, the phenomenon similar to that observed in the assimilation of magnesium and iron, has been observed in the assimilation of sulphur and phosphorus and in the formation of desoxyribonucleic acid and chlorophyll (1, 3, 4). It should also be remarked that the light cells are characterized in their stronger respiratory activity compared with dark cells (2). Needless to say, magnesium is an indispensable element in chlorophyll molecule and iron is a key element in the molecules of heme proteins which have been known to exist in close association with the photosynthetic apparatus in green cells. These considerations may give some clue to an understanding of the phenomenon that magnesium and iron are assimilated in larger measures by light cells than by dark cells.

The phenomenon that the content (in percent) of total ash dropped strikingly at the stages around L_1 is apparently due to the combined effects of the assimilation of magnesium, iron, sulphur and phosphorus, all of which are absorbed in lesser amounts in the growing stage than in the ripening stage.

ACKNOWLEDGEMENTS

This work was carried out as a part of the studies on the growth of *Chlorella* under the direction of Professor Hiroshi Tamiya, to whom the writer is indebted for his guidance. Thanks are also due to Dr. Eiji Hase for his helpful advice in the preparation of this paper. This work was aided by grants from the Ministry of Education and the Rockefeller Foundation. To these bodies we extend our grateful thanks.

SUMMARY

(1) By using the technique of synchronous culture of *Chlorella*, the changes in contents of potassium, magnesium and iron in successive developmental stages of algal cells were investigated.

(2) In percent of dry weight of cells, the content of potassium remained relatively constant throughout the life cycle, whereas the contents of magnesium and iron were lower at the growing stages (from the stage of "nascent dark cells" to the stage of "immature light cells") and higher at the ripening stage (the stages of light cells).

(3) A brief discussion was made in an attempt to relate the data obtained with earlier observations regarding the changes in contents of various other cell materials in the life cycle of *Chlorella* cells.

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STUDIES ON THE MICROORGANISMS FOUND IN THAI RICE AND BURMA RICE
*PART II. ON THE MICROFLORA OF BURMA RICE

HIROSHI IIZUKA

Division of Type Culture Collection, Institute of Applied Microbiology, University of Tokyo

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FORWORD

In this paper, the author is reporting the results of microbiological studies on the change of the microflora of deteriorated rice during his two-month visit to Burma, from December 1954 to January 1955[4], by the request of the Japanese Government and also some of his later investigations carried out in Tokyo on the samples collected during his trip[5].

1. Preparation for Investigation

With the view of performing ecological observations of microorganisms of the rice as influenced by the factors affecting not only the production and distribution processes but also the change of *Penicillium toxicarium* (=synonym *P. citreo-viride*), *P. citrinum* and *P. islandicum* which are known to produce toxic substances, the author has made some arrangment in advance to facilitate the quick isolation of microorganisms from collected samples. Equipments and materials necessary for research were shipped to Rangoon in order to make it possible to start work in time for the rice harvest season. The Burma Government, was kind enough to offer the author a room in the Department of Biology, University of Rangoon where isolation, culture, microscopic examination of microorganisms and determination of moisture etc. could be performed satisfactority.

2. Processes of Production, Treatment and Distribution of Burma Rice

Before starting with setting about sampling, of microorganisms the author made a survey on the prevailing conditions involved in production, refinement and distribution of Burma rice by investigating information supplied by the Ministry of National Planning and Religions Affairs, the Ministry of Finance State Agricultural Marketing Board (SAMB), Government of the Union of

* This was read at the General Meeting of the Agricultural Chemical Society of Japan held in Tokyo, March 30th 1955.

Burma and also other data[9-11]. In this paper, only a brief outline will be given.

The rice plantation fields are distributed in two main district. The first district is Lower Burma, where vast paddy fields lie along both banks of the River Irrawaddy, along the coast of Aracan and the North-Western coast of Malay Peninsula. The crop produced from this district is distributed from towns such as Rangoon, Moulmein, Bassein and Akyab. The second is Upper Burma, where irrigated paddy fields lie along both banks of the River Irrawaddy and Chindwin. The distribution centre of this district is the old capital Mandalay. Rangoon rice, which ranks with Thai or Siamese and Saigon rice in its history and productivity is largely produced in the first district. (Fig. 1.)

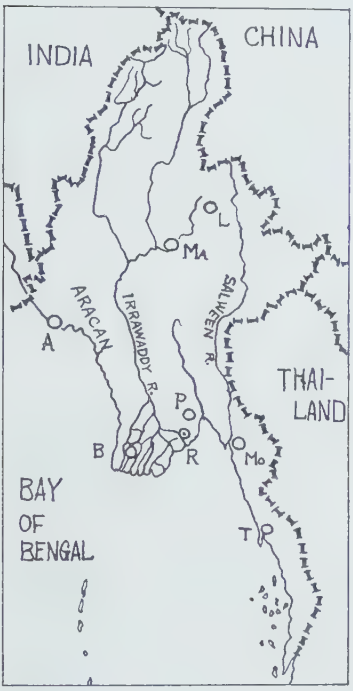

Fig. 1. Sketch of Burma
A: Akyab
B: Bassein
L: Lashio
M_A: Mandalay
M_O: Moulmein
P: Pegu
R: Rangoon
T: Tavoy

With the same exceptions according to districts or varieties of rice, seeding or bedding out of seeddling are made in the months of June and July. The harvest starts at the beginning of November just when the raining season is over and lasts three months. The procedures of refining the paddy, that is cutting, drying, thrashing, polishing etc. are still quite primitive. The rice, cut and left in the lower stalk about 30 cm above the gound, is packed in bundles and placed on the ground or path in the paddy field for 7~20 days and dried. Thrashing is performed in paddy fields after the harvest or in the farm yard by cattle turning round and round, tramping upon the rice bundles placed in a circle, 20~30 cm in height, the grains afterwards being collected. The collected paddy grains are stored in farm-houses or in a separate storehouse.

The chart of Fig. 2, gives an outline of the organization for distribution of rice in Burma. The State Agricultural Marketing Board (SAMB) which has its main office located at Rangoon and three branch offices at Moulmein, Bassein and Akyab makes purchases of paddy from farmers, cooperative sales corporations and brockers of paddy. According to the milling order of SAMB paddy is carried to the rice mill. The polished rice is packed in gunny bags of 100 Kg. net., each bag having its No. of producing zone, producing year,

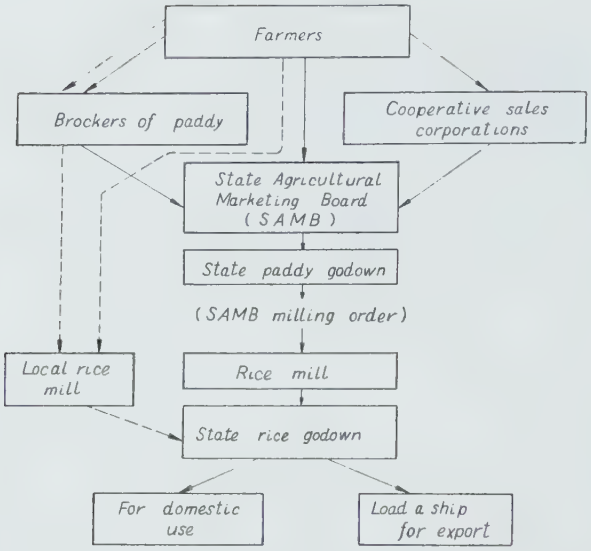

Fig. 2. Distrtbution processes of Burma Rice.

No. of milling order and No. of rice mill marked. Rice for export is shipped from the following ports Rangoon, Moulmein Bassein and Akyab.

3. Sampling of Specimens and Isolation of Microorganisms

The author has visited many places in the above-mentioned rice producing areas and collected soil, water, air, rice ears, straw, paddy etc. in the paddy field and its surroundings, and also collected paddy, polished rice, husks, dust, chip of wall, air, soil, etc. in farm-house, cargo boat, rice mill, godown and rice-shops. The sample was 300 in all.

Sampling of polished rice specimen from the stock stored in state godown was done in the following way: The rice was stored in the state godown, packed in gunny bags each containing 100 kg. in separate lots according to their grade, each in a pile of about twenty bags. About one thousand to five thousand bags of the same-grade rice were selected as test lots. From each lot, fifty bags were drawn from the top and side. From these bags the author drew samples and mixed them well so as to make a test sample which was subjected to culture test. Samples were drawn from fifty bags. Even when the number of bags contained in one lot was less than 1,000.

About twenty grams of rice was taken from 500 to 1,000 g. of rice samples at random in sterilized Erlenmeyer flasks, 250 ml in capacity, and each washed by shaking 20 times with about 10 ml of sterilized water. This is for the purpose of washing away the fungus spores attached to the grains. These operations should be conducted in a germ-free case. After repeating the above washing for twenty times, the kernels were placed one by one on

the Czapek agar medium in each test-tube up to 100 or 200 kernels and held for 7 to 12 days at 28° ±2°C; the species and the number of microorganisms developed on the kernels were then examined. In another test samples, microorganisms were isolated by the dilution method with Czapek agar and boullion agar medium and, for air plating culture in Petri dish was used on wash-fluid of the first washing and that of the twentieth washing. Because isolating conditions were considered of particular importance for this ecological investigation, isolation tests were made on the whole test samples upon returning to Japan by the following method: Polished rice kernels and paddy kernels and their husked rice kernels were placed in a shaker flask and after adding 150 ml sterilized water, washed by sufficient shaking to make the first wash-fluid. Then the sample was given two washings by shaking in 150 ml of 0.01% Tween 80 solution and eight more washings in 150 ml sterilized water to make the last wash-fluid. After the last washing, the sample was placed on three different agar media, Czapek, Koji extract and peptone glucose spread on Petri dish, by placing five sample kernels on each plate up to 100 kernels about each media and isolation was done by breeding at 28° and 37°C. At the same time, the first and last wash-fluids were subjected to isolation testing by means of plate culture at such dilutions as 10^{-3}, 10^{-4} and 10^{-5}, respectively under the same conditions as described above.

EXPERIMENTAL RESULTS

The results of the above tests are given in the varieties of the microorganisms and the number of their colonies found in various specimens as noted through Table 1 to Table 6 in case of Czapek medium at 28°. In these tables, P.i. indicates *Penicillium islandicum*, P.c.=*P. citrinum*, O.P.= other *Penicillia*, A=*Aspergillus*, (W. A.)=the number of white *Aspergillus* in *Aspergillus*, O.F.=Other moulds than *Penicillium* and *Aspergillus*, St.= *Streptomyces*, B=bacteria, Y=yeast, N=number of kernels not infested with internal microflora per 100 kernels.

The number of colonies of *P. islandicum* and *P. citrinum* are shown under separate items because these, together with *P. toxicarium* are specified by the Food Board and Ministry of Public Health of Japan as harmful moulds infesting the so-called deteriorated rice and are the subjects of inspection. Because *P. toxicarium* was not isolated from any of the specimen during the present tests, these tables have no special item for this mould. Of *Aspergillus*, the number of white *Aspergillus* in given separately because the number was characteristic and the mycological study on this moulds will be reported in a separate paper later. The data on the species of other microorganisms such as O.P., A., O.F., St., B, Y is too extensive to be listed in the tables appearing in the present paper. However, these data being highly important for the study of the change of microflora, will be outlined in explanation of ex-

perimental results. As a description of specimen SG stands for state godown, RM for refining mill.

Table 1. Microflora of two year-old crop rice.

Rice producing zone	Place of sampling	Grade	Microflora P.i.	P.c.	P.t.	O.P.	Asp.	O.F.	St.	Y.B.	N.
Rangoon B area	Kananto S.G.	Europe No. 3	0	0	0	0	12	4	3	8	76
	〃	〃	0	0	0	0	38	0	4	14	48
	〃	〃	0	0	0	0	16	4	4	2	78
Moulmein area	Mupun S.G.	Europe No. 3	0	0	0	0	25	1	0	55	18
Mandalay area	Botataung S.G.	Meedone KBQ	0	0	0	0	15	5	2	25	50

S.G.: State godown.

Table 2. Microflora of one year-old crop rice.

Rice producing zone	Place of sampling	Grade	Microflora P.i.	P.c.	P.t.	O.P.	Asp.	O.F.	St.	Y.B.	N.
Pegu area	Puzundaung S.G.	Ngastsein SMS	0	0	0	3	20	3	7	35	32
Moulmein area	New Mupun S.G.	Meedone	0	1	0	2	66	0	1	15	15
Moulmein area	Mupun S.G.	Ekarin	2	0	0	0	31	2	1	30	31
△Pegu area	Kemendine S.G.	Ngastsein SMS	0	0	0	2	0	3	1	2	85
Delta of the River Irrawaddy	Botataung S.G.	Meedone SQ	0	0	0	3	79	2	2	6	15
Myitkyina area	Botataung S.G.	Meedone KBQ	0	0	0	12	41	6	4	10	31
Maubin area	Fuming R.M.	Meedone Sabanet	0	1	0	2	14	3	9	14	56
Maubin area	Fuming R.M.	Meedone Sabanet	0	0	0	0	12	0	5	15	68
*Myaungmya area	Kemendine S.G.	Ngastsein SMS	0	1	0	1	100	0	1	3	3
*Moulmein area	New Mupun S.G.	Meedone	1	4	0	15	32	9	3	17	18

△: Re-polished rice.
*: Deteriorated rice of which deterioration can be recognized by visual inspection.
R.M.: Rice mill.

1. Microflora of Old Crop Rice (see Tables 1, 2 and Fig. 3)

The old crop rice refered here denotes rice of 1952–3 crop (Table 1) and 1953–4 crop (Table 2) or about one and two years old from the time of harvest stored as unhusked rice, and about three or four weeks after refination.

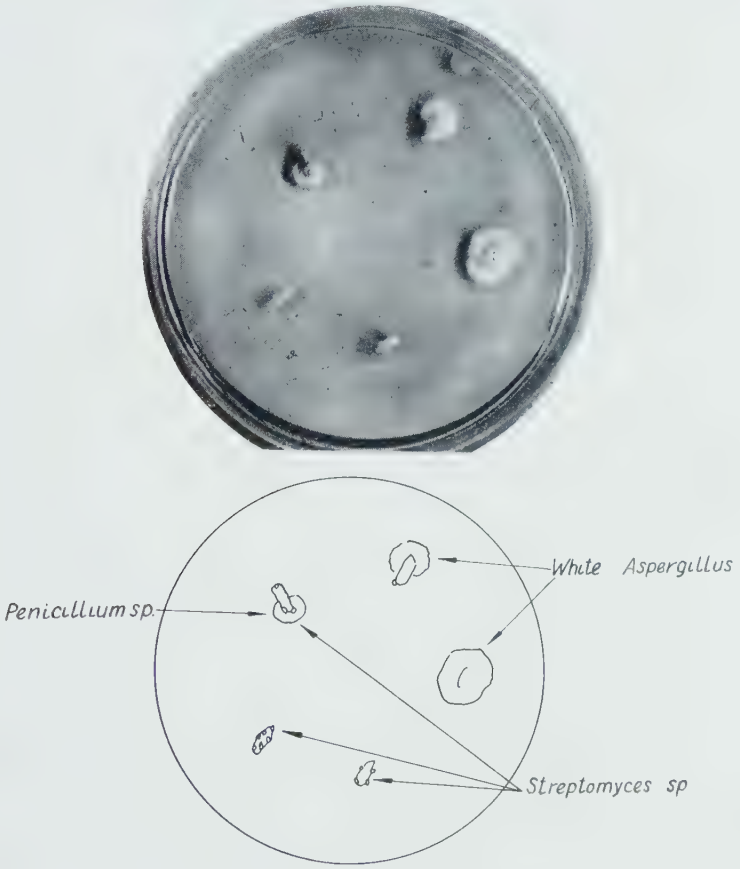

Fig. 3. The one year old crop rice kernels plated on potato agar at 28°C.

Some of the experimental results of rice of the 1952–3 crop are shown in Table 1. and summarized as follows.

(a) *Aspergillus* predominates among moulds and majority consists of *Asp. glaucus* group, *Asp. versicolor* group and white *Aspergillus*. (b) In item Y.B. in Table 1, 90% are represented by *Bacillus megatherium*. (c) Prievously, *Streptomyces* in rice has received little attention, but in the author's present investigation, this was found in many specimens and when isolation is done in the media after the addition of fungicides such as Eurocidin

(60 mg/1 l) or Kabicidin almost each old polished rice kernel will develop a considerable number of colonies on its surface.

Some of the results of rice of the 1953–4 crop are shown in Table 2. (a) The one year-old crop rice in general showed no wide difference as compared with the case of the two year-old crop rice. (b) In the freshly repolished one year-old crop rice sampled at refining mill (marked △ in Table 2), the number of non-infested kernels predominated, exceeding 80% and the number of moulds even in *Aspergillus*, was very small. (c) The common feature of the deteriorated rice of which deterioration can be recognized by visual inspection (marked * in Table 2), is that *Aspergillus* and *Penicillium* are found considerably than in the normal rice, giving a small number of non-infected kernels. (d) It was also found that the strains of *Streptomyces* isolated from these crop rice mostly consist of *S. diastaticus*, *S. Leben*, *Stessel* and *Keitt*, *S. californicus*, *S. albus* and *S. intermedium* and are considerably poisonous to the nervous system. When the mice were fed with food containing 10% of rice with any one of these strains grown on it, symptoms of disease appeared within several days, and they all died during the following several days[6].

2. Microflora of New Crop Rice (See Table 3 and Fig. 4)

This new crop rice indicates the 1954–55 crop rice, about one month old from the time of the harvest and a few days after refining. As shown in Table 3, in the microflora of these new crop rice a great difference was recognized from the case of old crop rice. (a) The new crop rice in general showed a wide difference when compared with the case of the old crop rice, the former gave a smaller number of *Aspergillus* and *Penicillium* about 0∼4, but a large number of the other moulds were about 5∼25, while the latter gave about 12∼79 colonies of *Aspergillus* and about 0∼6 colonies of the other moulds. (b) Moulds other than *Aspergillus* and *Penicillium* of the new crop rice were *Alternaria*, *Cepharosporium*, *Curvularia*, *Cladosporium*, etc. (c) *Aspergillus* of the new crop rice consists of white *Aspergillus*

Table 3. Microflora of new crop rice.

Place of sampling		Microflora Grade	P.i.	P.c.	P.t.	O.P.	Asp.	O.F.	St.	Y.B.	N.
Ku Aung Chin	R.M.	Ekarin SQ	0	0	0	0	1	25	7	39	45
Luxmi	R.M.	〃	0	0	0	0	2	9	0	13	77
Moulmein No. 90	R.M.	〃	0	0	0	1	0	12	0	33	55
Luxmi	R.M.	〃	0	0	0	0	4	6	7	16	67
New Mupun	R.M.	〃	0	0	0	0	3	7	4	14	74
Mupun	R.M.	〃	0	1	0	1	0	5	1	16	76
Mupun	R.M.	〃 No. 143	0	0	0	0	1	18	0	24	59

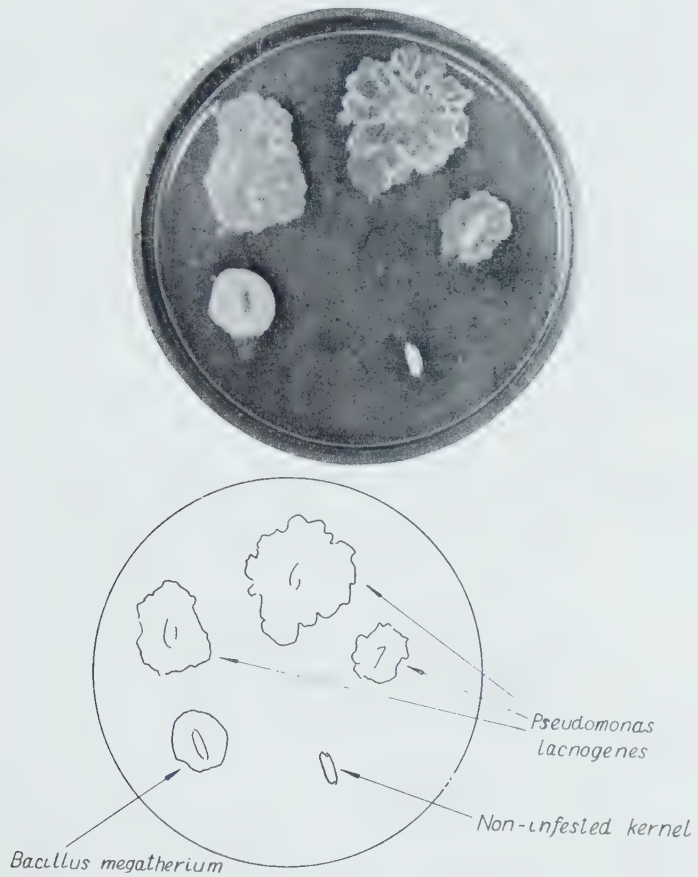

Fig. 4. The new crope rice kernels plated on Czapek agar at 28°C.

and *A. terreus* etc. (d) In item Y.B. in Table 3, the majority of Bacteria was the so-called Yellow *Pseudomonas* i.e: *P. lacnogenes*, *P. perlurida*, *P. xanthe*, *P. myxogenes*, etc.[1,2,3,7,8]

3. Microflora of unhusked Rice (see Table 4)

In Burma, rice is usually stored in the form of unhusked rice. The unhusked rice specimen kernels which were collected from rice ears immediately both in advance and after harvesting, or from rice bundle during sundrying, thrashing, and storing processes, were hand-unhusked with a disinfected pincette and separated into the husked rice kernels and husks, each of which were subjected to isolation tests in the same method as described above. Moisture of new crop unhusked rice was estimated as 15.7% by the electric resistance method measured at National Agricultural stations of Hmaubi. From the results of this test, the representative examples are

Table 4. Microflora of unhusked rice.

Grade	Microflora / Form	P.i.	P.c.	P.t.	O.P.	Asp.	O.F.	St.	Y.B.	N.
Ngastsein (in thrashing)	unhusked rice	2	0	0	5	6	89	0	5	0
	husked rice	0	0	0	2	0	68	1	32	0
Ngastsein (sifted out)	unhusked rice	4	7	0	21	23	50	0	5	0
	husked rice	0	0	0	0	0	62	0	34	6
Meedone Sabanet MBQ (commercial procucts)	unhusked rice	2	2	0	30	37	44	0	6	0
	husked rice	0	0	0	0	1	61	0	48	0
Ngastsein SMS (one year storaged)	unhusked rice	2	3	0	18	61	15	7	2	0
	husked rice	0	1	0	1	3	20	21	83	0
Par-boiled Paddy	unhusked rice	6	0	0	15	34	61	0	5	0
	husked rice	0	0	0	2	2	24	0	64	0

shown in Table 4. In the case of old crop unhusked rice the results were as follows. (a) In lining of microorganisms *Aspergillus* comes first followed by *Penicillium*, Mucorales and *Streptomyces*. Of these specimens, *Aspergillus* richly contained species of *A. tamarii*, *A. oryzae*. *A. fumigatus* and black *Aspergillus*. (b) Bacteria rich contained species with *Bacillus megatherium* and the so-called Yellow *Pseudomonas*. (c) Following this, experiments were performed in the same manner by washing and isolation in culture media on an unhusked but unpolished rice kernel still covered with pericarp which is called husked rice or hulled rice. The number of moulds decrease down to about 20% of the total, found in unhusked rice. Rice kernels in these form of the hulled rice showed active germination. (d) Almost all kernels of hulled rice showed germination, although the same period of time was not required. Furthermore, by viewing the relation of such germination and microorganisms, it was found that there is a higher percentage of germination in specimens containing the so-called Yellow *Pseudomonas* higher in number, than that of *Bacillus megatherium*. This was also found to be true in the case of unhusked rice. (e) In Par-boiled unhusked rice, a special type of rice with some peculiar odor prepared by boiling and drying in unhusked grains then refined by grinding which is also known to give smaller broken rice during grinding, no facts were found to justify the popular belief that this type of rice is less liable to contract microbiological pollution.

In the case of new crops, husked rice gave very few *Aspergillus* and

Penicillium in moulds the majority of which consisted of *Alternalia, Curvularia, Cepharosporium, Helminthosporium, Chaetomium, Cladosporium* and other non-sporeforming moulds. A fairly large number of bacteria was detected the majority of it was being the so-called Yellow *Pseudomonas*.

4. The Change of Microflora found in New Crop Rice During Refining Process (see Table 5)

In refining mills in Burma, unhusked rice is refined by grinding into highly-polished rice in a one-process, during which sample bran contents are removed from the grains. For the purpose of the author's investigation, a new crop was sent to the refining mill for grinding and specimens were collected at each stage of the refining process conducted in Moulmein area. These specimens were subjected to investigation about forty hours later at Rangoon University. The results are listed in Table 5. and are summarized as follows:

Table 5. The change of microflora in new unhusked rice during refining process.

	Mill and Specimen	Microflora / Form	P.i.	P.c.	P.t.	O.P.	Asp.	O.F.	St.	Y.B.	N.
Moulmein area	Luxmi R.M. No. 1	unhusked rice	0	0	0	15	17	74	0	10	0
	Ekarin SQ	husked rice	0	1	0	4	2	23	0	64	1
		polished rice	0	0	0	0	4	6	7	16	67
	Luxmi R.M. No. 2	unhusked rice	3	0	0	32	17	55	0	20	0
	Ekarin SQ	husked rice	0	1	0	14	11	41	0	41	1
		polished rise	0	0	0	0	2	9	0	13	77
	Ku Aung Chin R.M.	unhusked rice	0	1	0	18	0	98	0	11	0
	Ekarin (for domestic use.)	husked rice	0	1	0	0	0	75	0	69	0
		polished rise	1	0	0	0	1	25	7	39	45
	Moulmein No. 90 R.M.	unhusked rise	1	3	0	28	14	52	0	2	0
	Ekarin SQ	husked rice	0	2	0	6	1	35	5	48	2
		polished rice	0	0	0	1	0	12	0	33	55

In the freshly-polished new rice kernel after the removal of husks (inner- and outer-glume), bran layer (pericarp, endosperm) and sometimes a part of the aleuron layer, the microorganisms showed a significant decrease both in number and the so-called Yellow *Pseudomonas*, in item Y.B. in Table 5, and some moulds found to invade rice during cultivation, in item O.F. in Table 5. The number of the so-called Yellow *Pseudomonas* was 10^7 per 1 g of this husked rice.

5. Microflora of Rice for Local Consumption (see Table 6)

In order to investigate about microflora of the rice daily consumed by Burmese people, specimens were collected from the stocks of retailers, consumers and tested. The results are partly shown in Table 6. In the

Table 6. Microflora of rice for local consumption.

Samples \ Microflora	P.i.	P.c.	P.t.	O.P.	Asp.	O.F.	St.	Y.B.	N.
2nd class, 38 Kyat per 1 bag	0	0	0	4	7	1	1	5	83
1st class, 45 Kyat per 1 bag	0	0	0	0	3	4	13	9	74
Special class	1	0	0	0	0	0	0	14	86
Sabanet: in use of the Japanese Embassy staff, for domestical purposes	0	0	0	2	15	1	1	1	80
N. Co.: for domestic use.	0	0	0	1	0	15	0	15	70
*Glutinous rice: N. Co. for domestic use.	0	0	0	3	48	1	1	21	21
Sabanet: M. Co. for domestic use.	0	0	0	1	4	3	2	6	84
Sabanet: D. Co. for domestic use.	0	0	0	6	14	0	1	0	75
Sabanet: Kitchen of University of Rangoon	0	0	0	2	21	2	0	9	66

* *Aspergillus* predominate among other moulds and the majority consists of white *Aspergillus*.

glutinous rice (marked * in Table 6) particularly, many white *Aspergillus* were frequently detected from normal polished rice and such a fact was also verified in Thailand with the new crop polished glutinous rice[1-3].

SUMMARY

In investigating the causes of deterioration of cereal grains under the influences of microorganisms, the author has held the view that the most important approach is to trace the movements of microflora in an ecological heterogenous system, termed cereal grain. Such microbiological studies on the cause of deteriorated rice, have been carried out in the same light. Results of this investigation are summarized as folllows.

(1) From a standpoint of microbiology, rice will be stored in unhusked form, which affords far more safety than in the polished form in Burma.

(2) In regard of the movements of microflora during the storage period of unhusked rice in Burma, new crop rice gives very few *Aspergillus* and *Penicillium* and many so-called Yellow *Pseudomonas*, i.e: *Pseudomonas lacnogenes*, *P. perlurida*, *P. xanthe*, *P. myxogenes*, etc. however, in normal rice after it is stored during raining season in the form of unhusked rice, the number of *Aspergillus*, i.e: white *Aspergillus*, *A. versicolor*, *A. fumigatus*, *A. glaucus* group, etc. *Penicillium*, i.e; *P. citrinum*, *P. notatum*, *P. oxalicum*,

P. commune, *P. cyclopium*, *P. islandcium*, *P. rugulosum*, *P. chrysogenum*, etc. and *Streptomyces* increase.

(3) There is some rice of which deterioration can be recognized by visual inspection. The common feature of this deteriorated rice is that considerably more amounts of *Aspergillus*, *Penicillium* and *Streptomyces* are contained than in normal rice.

(4) Up to the present no attention has been paid to *Streptomyces* found in rice. However, from the author's investigations many of these were found in almost every specimen of normal rice. Furthermore, these *Streptomyces* of rice mainly consist of *S. diastaticus*, *S. leben*, *Stessel* and *Keitt*, *S. carifornicus*, *S. albus*, *S. intermedium* etc. It was further found that some of these *Streptomyces* possesses a considerably poisonous function when mice were fed with food containing rice with any one of these strains grown on.

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OXIDATIVE FERMENTATION OF D-RIBOSE BY *LACTOBACILLUS PLANTARUM* NO. 11
(Preliminary Report)

SAKUZO FUKUI and AKIRA ÔI

Division of Zymomycology, The Institute of Applied Microbiology, University of Tokyo

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Two different pathways of anaerobic pentose metabolism have been reported by application of lactic acid bacteria as the microorganism[1),(2)]. Recently, in concern of the aerobic pentose metabolism in *Pseudomonas saccharophila* the following schemes were proposed by Doudoroff and his coworkers[3),(4)]:

$$\text{D- or L- Arabinose} \xrightarrow[(\text{DPN})]{-2\text{H}} \text{Arabonolactone} \xrightarrow{+H_2O} \text{Arabonate} \longrightarrow \begin{matrix}\text{Pyruvate+Glycolate} \\ \text{or} \\ \alpha\text{-Ketoglutarate}\end{matrix}$$

According to this scheme, no phosphorylating process was involved but in the case of D-arabinose metabolism it was found that D-arabonate dehydrase, catalized the reaction

D-Arabonate ⟶ 2-Keto, 3-deoxy-arabonate,

was presented in the fermentation. Consequently, D-arabinose was introduced into equimolar pyruvate and glycolate and L-arabinose into α-ketoglutarate.

This paper deals with the oxidative D-ribose metabolizing pathway caused by *Lactobacillus plantarum* No. 11.

The intact cells of *L. plantarum* No. 11 grown in a medium containing D-ribose or D-gluconate could aerobically ferment D-ribose according to the following new fermentative equation,

$$C_5H_{10}O_5+O_2 \longrightarrow 2CH_3COOH+CO_2+H_2O$$

In this case, acetate and carbondioxide were produced from D-ribose as main products, but a significant amount of lactate could not be observed as presented in Table 1. Through the fermentation any accumulation of pyruvate and α-ketoglutarate was not detected. The gas balances which were observed on the reaction upon various pentoses and pentonates by the cells are listed

in Table 2. When D-ribose was used as substrate, equimolar O_2-consumption and CO_2-evolution were observed. D-Ribulose prepared from D-arabinose was fermented as a case of D-ribose, and D-ribonate synthesized from D-ribose was metabolized in a gas balance of O_2-uptake/CO_2-evolution=1.8/2.9. While other substrates tested here were certainly unattacked. This organism was found to possess abilities of dehydrogenation toward D-ribose and D-ribonate

Table 1. Aerobic fermentation of D-ribose by the cells of *Lactobacillus plantarum* No. 11.

Initial D-ribose	Consumed D-ribose	Products		
		Acetic acid	Lactic acid	Pyruvic acid+α-ketoglutaric acid
μM	μM	μM	μM	μM
3,320	2,301	4,013	261	Trace

For the purpose of obtaining the D-ribose fermentable cells of *Lactobacillus plantarum* No. 11, the following medium was used: 1% D-gluconate, 1% fish-meat extract, 1% peptone, 0.5% NaCl, and 0.1% Difco's yeast extract in tap water. The cells of this organism were harvested by centrifugation from the culture of 1,000 ml after 20 hours incubation at 30°C. The yellowish cells thus obtained gave a yield of approximately 300 mg. Aerobic D-ribose fermentation was carried out on a reciprocal shaker at 30°C incubation for 19 hours.

Reaction medium: 300 mg cells, 498.0 mg D-ribose in M/100 phosphate buffer, pH 7.0–7.2, 50 ml.

Determination of products: Acetate, lactate and keto acids were determined by the methods of steamdistillation, Barker-Summerson and Cavallini respectively.

Table 2. Gas balances in aerobic D-ribose, D-ribonate, D-ribulose and D-lactate metabolism by the cells of *Lactobacillus plantarum* No. 11.

Substrate	O_2-Uptake μM	CO_2-Evolution μM	Formed lactate μM	Consumed substrate μM
D-Ribose	4.1	4.2	Trace	4.0
D-Ribonate	1.8	2.9	Trace	not-determ.
D-Ribulose	1.2	1.3	Trace	1.5
D-Lactate	0	0	—	0 (Trace)

D-Xylose, L-xylose, L-arabinose, D-arabinose, L-ribulose, D-xylulose, D-xylonate and L-arabonate were unable to be metabolized.

Reaction medium: Cell suspension 0.5 ml (corresponded to 10 mg dried cell).
M/15 Phosphate buffer, pH 7.0–7.2, 0.5 ml.
Substrate, 4 μM, plus distilled water, 1.0 ml.
Total, 2.0 ml.

Reaction period: 1 hour.

Reaction temperature: 35°C.

Gas balances were measured by manometric technique.

as shown in Table 3. No accumulation of D-ribonolactone or its derivate as the first product of the enzymic dehydrogenation in the ribose metabolism could be detected, even when hydroxylamine was added to the reaction medium as a trapping agent. But phospho-D-ribonate could be detected as hydroxyamic acid by the method of Hestrin[5]. The spectrophotometric observation on the dehydrogenating action upon D-ribose by using opal glass in a Beckman spectrophotometer (model DK-2) have demonstrated that the alteration of absorption spectrum at 390 mμ and 453 mμ which presented in the cells was occurred with partially disappearence by addition of D-ribose or D-ribonate as substrate (Fig. 1). The reversed reaction of it was observed

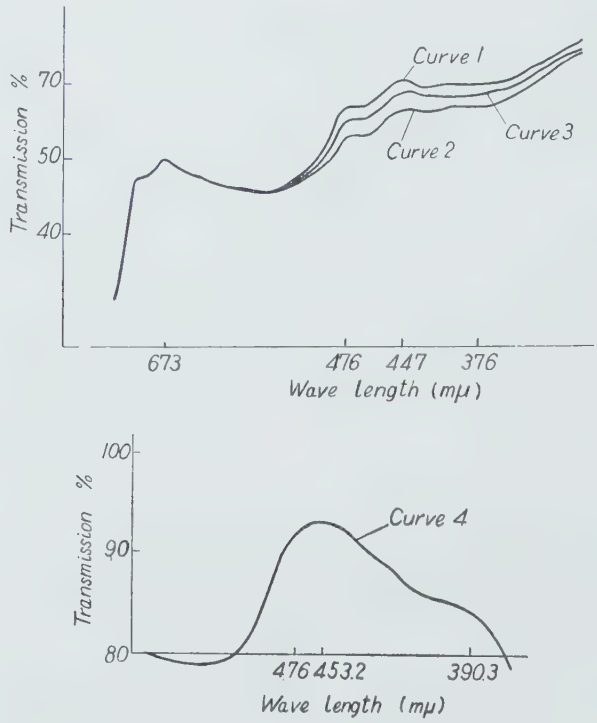


Fig. 1. Absorption spectrums of cell suspensions of *Lactobacillus plantarum* No. 11.

Absorption spectrums of cell suspensions measured by the use of opal glass in the Beckman spectrophotometer model DK-2.

Curve 1: Cells suspended in M/15 phosphate buffer, pH 7.0. Curve 2: D-Ribose 10 μM added to a cell suspension and spectrum observed after 10 minutes incubation at 28°C. Curve 3: Aerated reaction medium after 10 minutes incubation with 10 μM D-ribose. Curve 4: Difference of Curve 1 and 2, namely the spectrum of the cell suspension observed by using the reaction mixture of Curve 2 as a reference.

Table 3. Dehydrogenation of various sugars by the cells of *Lactobacillus plantarum* No. 11.

Substrate	Glucose	Gluconate	D-Ribose	L-, and D-Arabinose	D-Xylose	D-Ribulose	D-Ribonate
Decoloring time min.	1	2	5.5	>60	>60	15	13

The dehydrogenating ability is expressed as decoloring time, minutes, for 0.2 μM 2, 6-dichloro-diphenyl-indophenol.

Reaction medium: Cell suspension 0.1 ml (corresponding to 0.5 mg dried cell).
M/15 Phophate buffer, pH 7.0–7.2, 0.1 ml.
Substrate 5 μM, 0.1 ml.

Reaction temperature: 28°C.

Table 4. Dehydrogenation of various sugars by the cell-free preparation.

Addition \ Substrate	Glucose	G-6-P	FDP	6-P-G	D-Ribose	D-Ribulose	D-Ribonate	D-, and L-Lactate
None	24	14	>120	7	>120	>120	>120	>60
ATP	10	13	>120	7	14	43	48	>60

Dehydrogenating ability is expressed as Table 3.

Reaction medium: Cell-free preparation 0.1 ml.
2, 6-Dichloro-diphenyl-indophenol 0.2 μM, 0.1 ml.
Substrate 5 μM, 0.1 ml.
ATP 0.3 μM, 0.1 ml.

Reaction temperature: 28°C.

Ten ml of cell free preparation was obtained from the cell paste (corresponding to 310 mg) by grinding and extracting.

Table 5. Effects of DPN, TPN and ATP on the dehydrogenating activities toward D-ribose and D-ribonate by the dialyzed enzyme.

Addition	DPN	+	+	−	−	+
	TPN	+	−	+	−	+
Substrate	ATP	+	+	+	+	−
D-Ribose		13	19	13	28	>120
D-Ribonate		10	11	19	29	>120

Decoloring time in minutes.

Reaction medium: Dialyzed enzyme 0.1 ml.
Substrate 5 μM, 0.1 ml.
M/15 Phosphate buffer, pH 7.0–7.2, 0.1 ml.
DPN, TPN and ATP, 0.3 μM, each 0.1 ml.
2, 6-Dichloro-diphenyl-indophenol 0.2 μM, 0.1 ml.

Reaction temperature: 30–3°C.

by aeration, and was not affected by KCN in a concentration of 5×10^{-3} M at final. As described above, the reversible specific changes on absorption spectrum evidently pointed out the occurrence of hydrogenation and dehydrogenation in the flavin-enzyme molecule. Then it is considered that this autooxidizable flavoprotein contained in the cells may be functioning as a terminal oxidase in the oxidative D-ribose metabolism.

The cell-free preparation which contained the whole system of this

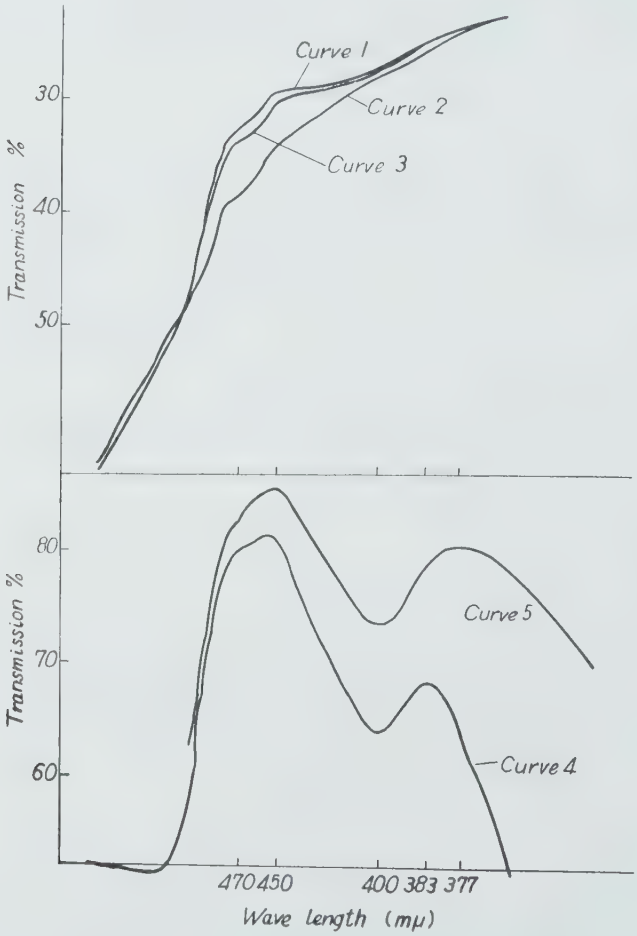


Fig. 2. Absorption spectrums of the cell-free preparation.

Curve 1: The cell-free system and ATP added cell free system. Curve 2: D-Ribose 10 μM added to the ATP contained system and spectrum observed after 30 minutes incubation in the photometer's vessel. Curve 3: Aerated reaction mixture of Curve 2. Curve 4: Difference of Curve 1 and 2. Curve FAD only.

pathway was obtained by grinding the D-ribose or D-gluconate adapted cells with sea-sand and extracting with M/15 phosphate buffer, pH 7.0–7.2, for 30 minutes at room temperature. This preparation was capable of dehydrogenating D-ribose, D-ribonate and D-ribulose by addition of ATP as presented in Table 4 and 5.

According to the findings mentioned above, the following new scheme may be proposed for the oxidative D-ribose metabolizing pathway by *Lactobacillus plantarum* No. 11:

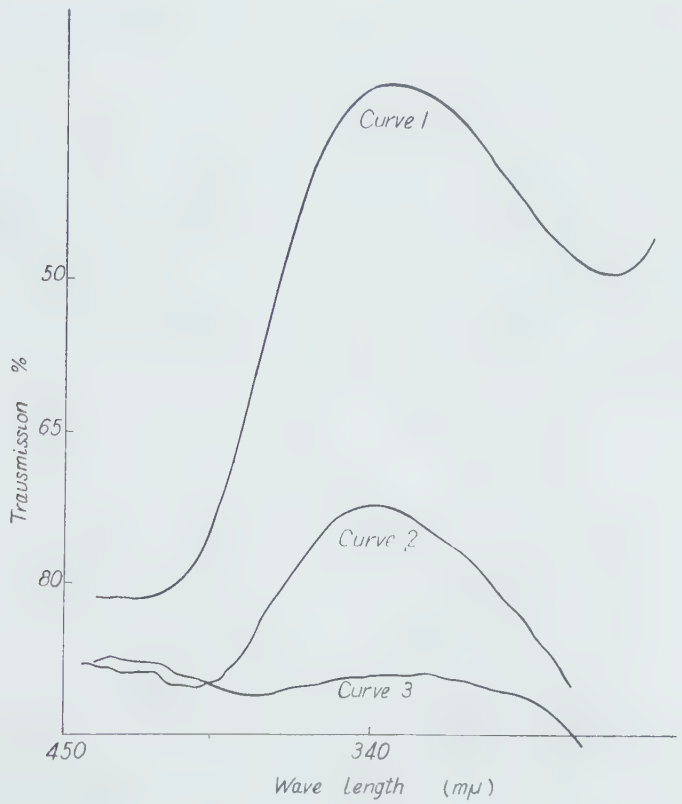
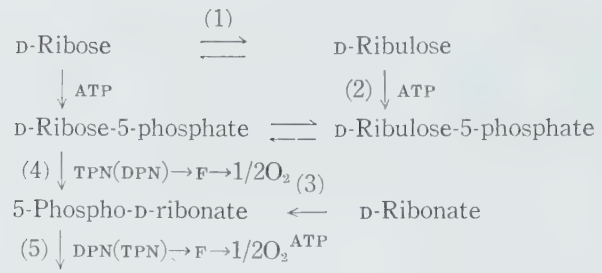


Fig. 3. Oxidation of TPNH by the cell-free preparation.

TPNH: Prepared from TPN by the method of $Na_2S_2O_4$ reduction.

Curve 1: TPNH 0.5 μM in 3.5 l distilled water. (reference H_2O) Curve 2: 0 time incubation, reaction medium is TPNH 0.5 μM, cell-free preparation 0.5 ml plus distilled water 3.0 ml. Spectrum observed by using enzyme preparation 0.5 ml plus distilled water as a reference. Curve 3: After 10 minutes incubation at 25°C, spectrum observed as Curve 2.

Oxidation of DPNH was also observed under same condition.



(1), D-Ribose isomerase; (2), D-Ribulokinase; (3), D-Ribonokinase; (4), D-Ribose-5-phosphate dehydrogenase; (5), Oxidative 5-phospho-D-ribonate splitting system; F, Flavin enzyme.

The cleavage reaction of D-ribonate could be observed in the presence of ATP and DPN or TPN by using the cell-free system. The details are now in progress. The flavin enzyme included in the system contained FAD as a prosthetic group and was reduced by DPNH and TPNH (Fig. 2, 3 and 4). Reversed reaction of this was readily occurred by contact with molecular oxygen (Fig. 2) and was not affected by KCN in a concentration of 10^{-2} M. Therefore, in the oxidative D-ribose and D-ribonate metabolism, hydrogen atoms in TPNH and DPNH introduced from substrates might be transferred to molecular oxygen through the flavoprotein.

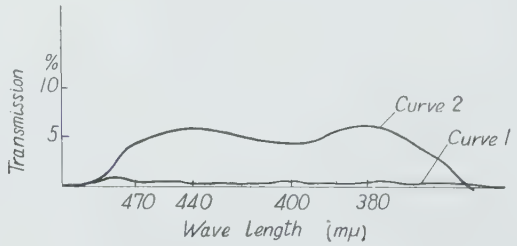


Fig. 4. Reduction of flavin-enzyme by DPNH.

DPNH: Prepared from DPN by the same manner in the case of TPNH. Spectrums observed by using cell-free preparation as a reference.

Curve 1: DPNH no addition. Curve 2: DPNH 0.5 μM was added to the system of Curve 1, and gently mixed. After 5 minutes incubation spectrum observed.

Same reduction of the flavin-enzyme by TPNH was able to be demonstrated.

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L-LYSINE PRODUCTION USING MICROBIAL AUXOTROPH

(Preliminary report)

SHUKUO KINOSHITA, KIYOSHI NAKAYAMA and SOHEI KITADA

Tokyo Research Laboratory, Kyowa Fermentation Industry Company

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The deficiency of L-lysine in cereal protein has become quite an important problem in human nutrition during the last few years. This problem is considered to be more serious for people in oriental countries owing to their high dependence on cereal foods.

Based upon this background the production of L-lysine has been undertaken by some investigators[1-3].

In previous papers, one of the authors has reported on this success in producing L-glutamic acid[4] and L-ornithine[5] by fermentative processes using a newly identified species named *Micrococcus glutamicus*.[6]

In the course of mutation studies of *M. glutamicus* using U.V. and Co^{60}, various types of auxotrophic mutants were isolated. Among these homoserine or both methionine and threonine-requiring mutants (*M. glutamicus* No. 613–1 and *M. glutamicus* No. 614–5) was found to be able to accumulate large amounts of L-lysine in the culture broth. The chief ingredients in the medium used in the above experiments were 2.5% glucose, 0.5% NH_4Cl, 0.2% NZ-amine and mineral salts. Although L-lysine was contained in the medium itself, the final amount of L-lysine far exceeded that of the initial amount; therefore it was self evident that L-lysine was produced by the organism directly from glucose and nitrogenous material.

A Similar phenomenon was also observed on auxotrophic strains of *M. glutamicus* which required methionine or homocysteine (*M. glutamicus* No. 631–2), methionine (*M. glutamicus* No. 602) and both leucine and isoleucine (*M. glutamicus* No. 613–611). In case of homoserine or methionine-requiring strains of *E. coli*, L-lysine was also produced.

L-Lysine–HCl crystal obtained from culture broth by an ion exchange method melted at 262–3°C. Anal. Calculated for $C_6H_{14}O_2N_2 \cdot HCl$: C, 39.42; H, 8.28; N, 15.33. Found: C, 39.33; H, 8.43; N, 15.17. $[\alpha]_D^{13°} = +24.9$ (C=2, 6N HCl). These results were in good accordance with values of L-lysine found in the literature. The expected amount of CO_2 was also evolved from a sample solution by a L-lysine decarboxylase preparation of *Bacterium cadaveris* NCTC 6758.[7]

The outstanding feature of the L-lysine fermentation using *M. glutamicus* No. 613–1 was that L-lysine is the only main product and, such by-

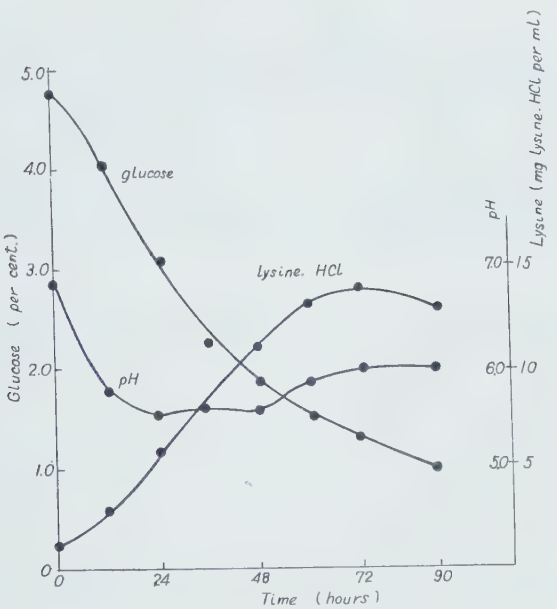

Fig. 1. Chemical change in lysine fermentation.
Microorganism: *Micrococcus glutamicus* 613-1 (homoserine⁻).
Medium composition: glucose 5.0, NH_4Cl 1.0, NZ-amine 0.4, KH_2PO_4 0.05, K_2HPO_4 0.05, $MgSO_4 \cdot 7H_2O$ 0.025, $FeSO_4 \cdot 7H_2O$ 0.001, $MnSO_4 \cdot 4H_2O$ 0.001, Yeast extract 0.1, $CaCO_3$ 0.5 (per cent.)
Temperature: 28°C.

products as organic acids and other amino acids are practically negligible.

In Fig. 1, an example of fermentation is shown. The amount of L-lysine produced from consumed glucose was as high as 29% on weight basis.

It may thus be said that the fermentative production of L-lysine will possibly become the most potent industrial method.

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Abstract

Reports of studies conducted by members of the Institute of Applied Microbiology, University of Tokyo, published elsewhere are abstracted.

Nature, **178**, 706 (1956)

A high frequency of hetrozygous and somatic recombination produced by ultraviolet light in imperfect fungi

By Chiyoko ISHITANI

Division of Microbial Genetics.

When heterocaryotic conidia of *Aspergillus oryzae* or *Asp. sojae* were exposed to ultraviolet light, the frequency of diploidization was increased from 10^{-7} to 10^{-2} at a survival of 10^{-2}. The heterocaryons were produced between a white leucineless and a yellow methionineless mutant, and the heterozygous diploids were diagnosed under the following criteria; (1) green prototrophic in phenotype, (2) more stable than the heterocayons, (3) segregate recombinants in a low frequency, (4) DNA content per each nucleus is about twice of that in haploid nucleus.

Ultraviolet light was also effective in stimulating somatic segregation. When conidia from a green diploid colony were irradiated with different doses, there was a decrease from 9 to 75 per cent in the green colonies formed upon planting.

Japanese J. of Genetics, **32**, (1956) 147–152

Gene controlled adenine dependence epistatic to color productioe in *Saccharomyces*

(in English)

By Hiuga SAITO and Toshiaki TAKAHASHI

Division of Microbial Genetics.

Apparent non-Mendelian segregation encountered in a cross between adenine independent and adenine dependent haploid strains is explicable by postulating a multiple genetic control of adenine formation. One of these genes, which arose by spontaneous mutation in an originally pink adenine dependent culture, is presumed to block synthesis prior to those already present in the stock. The postulate Ad_3 mulation is not linked with the other adenine genes, which, when mutant, cause color production, but it is epistatic to them.

Experimental Cell Research, **10**, (1956) 737–740

The relation of DNA content to cell size in *Aspergillus*

By Chiyoko ISHITANI, Kazuo Uchida and Yonosuke IKEDA

Division of Microbial Genetics.

By measuring the total DNA content in haploid, diploid, and heterocaryotic conidia of *Aspergillus sojae* and by counting the number of nuclei in each conidium, DNA content per nucleus was calculated. The cell size and the total DNA content of both haploid and diploid conidia are equal, but the number of nuclei in diploid conidia is about half of that in haploid conidia, persisting the validity that diploid nuclei comprise twice as much DNA of that in haploid nuclei. In contrast to the haploid conidium that contain four nuclei in average, the heterocaryotic conidium contain from five to eight nuclei, and the cell size becomes larger in accordance with the increase in number of nuclei in one conidium. The constency of the DNA-cytoplasmic ratio was thus evidenced even in the heterocaryons.

Bull. Agr. Chem. Soc. Japan, **19**, (1955) 221–227

Paper electrophoretic studies on the diastatic enzymes of molds

I. Electrophoretic technique and the behavior of diastatic enzymes observed on papers

(in English)

By Tadahiko ANDO and Yonosuke IKEDA

Division of Microbial Genetics.

A detailed procedure for the separation of diastatic enzymes of molds was established,

which was based on their electrophoretic behavior on a paper strip. For the detection of enzymes, a bioautographical method was newly devised. Comparing the enzymatic images appearing on the paper strip, mold enzyme systems were classified into five categories. Group 1—*Aspergillus oryzae*, *Asp. sojae*, and *Asp. glaucus*. Group 2—*Asp. usamii*. Group 3—*Asp. awamori*, and *Asp. inui*. Group 4—*Rhyzopus tonkiensis* and *Rh. javanicus*. Group 5—*Mucor spinescens*, *Rh. peka*, and *Penicillium chrysogenum*.

Microbial Genetics Bulletin, **15**, (1957) 25-26

Genetic recombination in *Streptomyces griseoflavus*

By Hiuga SAITO

Division of Microbial Genetics.

Apparent genetic recombination was evidenced in cross tests between various kinds of biochemical mutants of *Streptomyces griseoflavus*. Among the six combinations that gave positive results, only the cross between a methionine-adenineless and a niacine-histidineless strain yielded complementary products, i.e., adenine-histidineless and methionine-niacineless. Some combinations were positive in heterocaryon formation and negative in genetic recombination. The selection of fitter type recombinants in the course of nuclear division after recombination and the possible compatibility factor(s) of *Streptomyces* species are discussed.

Experientia, **13**, (1957) 473

Evidences for the bipartite or diploid nuclei in conidia of *Streptomyces griseoflavus*

By Hiuga SAITO and Yonosuke IKEDA

Division of Microbial Genetics.

Evidences for the bipartite or diploid nuclei in conidia of *Streptomyces griseflavus* were produced in four ways. (1) Cytologically, the nuclei in an early stage of conidia formation are dumbbell-like, and afterwards turn into one unit. (2) Radiokinetic analysis did give rise to "two hit curve". (3) Biochemical mutants were recovered in a high frequency when irradiated conidia were preincubated on a complete medium prior to application of the filtration technique. (4) Revertants from an auxotrophic mutant yielded heterogeneous colonies carrying both prototrophic and auxotrophic conidia.

Kagaku, **27**, (1957) 604-609

The life cycle and heredity of koji molds (*Aspergillus oryzae* and *Asp. sojae*)

(in Japanese)

By Yonosuke IKEDA

Division of Microbial Genetics.

This paper consists of six paragraphs; (1) induction of biochemical mutants, (2) production of heterocaryons, (3) production of heterozygous diploids, (4) segregation of recombinants from heterozygous diploids, (5) production of interspecific hybrids, and (6) a general survey on the parasexual life cycle of koji molds. Application of genetic techniques to the improvment of industrial strains is suggested.

J. Agr. Chem. Soc. Japan, **30**, (1956) 1-7

Studies on the oxidative bacteria. Part 13.

Dehydrogenating activities of intact resting cells of several oxidative bacteria to glucose, gluconate and ketogluconate

By Toshinobu ASAI and Haruyoshi MUROOKA

Division of Physiology.

Oxygen uptake with glucose, gluconate and ketogluconate by intact resting cells of *Pseudomonas fluorescens liquefaciens*, *Pseudomonas fluorescens* 40F, *Acetobacter suboxydans* ATCC 621 and *Gluconoacetobacter roseus* was manometrically determined.

Net oxygen consumptions of glucose, gluconate, and 2-ketogluconate by *P. fluorescens liq.* were exceeded more than 6 atom/molecule of substrate and it can be concluded that glucose was dissimilated via gluconate and 2-ketogluconate.

However, *P. fluorescens* 40F did not show any oxygen uptake with 2-ketogluconate, in spite of having oxidative activity on glucose and gluconate, and 2-ketogluconate was not detected in the culture fluid of gluconate, thus suggesting that this strain may not participate in the oxidative pathway via 2-ketogluconate.

The optimal pH of oxidation of glucose and gluconate by *P. fluorescens liq.* and *P. fluorescens* 40F lie at a more alkaline side than those of *A. suboxydans* 621 and *G. roseus*, namely the former strains have their optimum at 7.0 to each substrates, whereas in the latter two, optimal pH were found to be 5.0 to glucose and 4.0 to gluconate respectively.

Oxygen uptake with 5-ketogluconate was recognized only in case of *P. fluorescens liq.*. It was suggested that this strain might assume a pathway via 5-ketogluconate. Any remarkable oxygen uptake with 5-ketogluconate was not observed in *P. fluorescens* 40F, *A. suboxydans* ATCC 621 and *G. roseus*. However, in the presence of a very small amount of glucose there occurred a strong oxygen uptake with 5-ketogluconate restrictively in the case of *A. suboxydans* ATCC 621.

J. Agr. Chem. Soc. Japan, **31**, (1957) 225–229

Studies on oxidative fermentation. Part 14.

Catabolism of K-5-ketogluconate. 1

By Toshinobu Asai, Haruyoshi Murooka and Yukashi Kobayashi

Division of Physiology.

K-5-Ketogluconate was oxidized by intact resting cells of *Acetobacter suboxydans* ATCC 621 in a phosphate buffer (pH 5.0) with a small amount of glucose. Reducing power of the reaction liquid showed a 67% descrease in 24 hours. The reaction mixture was centrifuged and the supernatant was passed through a column of cation exchange resin (Amberlite IR-120) and extracted with ether. Acetic, succinic, α-ketoglutaric and glycolic acids were detected and identified in the ether soluble part. Though the total amount of these substances was only about 5% of added K-5-ketogluconate, it was ascertained that these substances were formed neither by the spontaneous breakdown of K-5-ketogluconate, nor by the oxidation of a small amount of added glucose, nor by the decomposition of cells, but by the enzymatic reaction of K-5-ketogluconate with the intact resting cells of *A. suboxydans* ATCC 621.

Bull. Agr. Chem. Soc. Japan, **21** (2) 134 (1957)
Hakko-Kyôkai-Shi, **15** (9) 371 (1957)

On L-glutamic acid fermentation

By Toshinobu Asai, Kô Aida and Kunio Ôishi

Division of Physiology.

Bacteria which accumulate L-glutamic acid in media consisting of glucose and inorganic ammonium salts were isolated from various natural sources. Many kinds of bacteria such as *Micrococcus*, *Bacillus*, *Streptococcus*, *Xanthomonas*, *Pseudomonas*, *Aeromonas*, *Serratia*, *Esherichia* and *Aerobacter*, fungi such as *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor* and other microorganisms including *Streptomyces* and fungi imperfecti were found to accumulate L-glutamic acid.

The strains belonging to the genus *Micrococcus* and *Bacillus* showed a high level of accumulation of L-glutamic acid among these microorganisms. A strain which was identified as *Micrococcus varians* had the most powerful ability to accumulate L-glutamate. The yield of L-glutamic acid was 28.2% of the glucose added.

Some strains which accumulated L-alanine, L-aspartic acid, L-leucine and/or L-valine were also isolated.

The culture condition of L-glutamate accumulation was investigated, from which it was found that ammonium chloride was the most suitable nitrogen source and submerged culture was more effective than surface culture.

In the course of L-glutamic acid fermentation by *Micrococcus varians*, a small amount of L-alanine, L-aspartic acid, α-ketoglutaric acid, pyruvic acid and succinic acid were also found to be formed besides L-glutamic acid. Judging from the kind of these products and the time of their formation, the chemical pathway of L-glutamate formation was suggested as follows:

glucose⟶pyruvate⟶α-ketoglutarate ⟶L glutamic acid.

Bull. Agr. Chem. Soc. Japan, **22**, (1958) 1–5

On the metabolism of D-glucuronic acid by *Pseudomonas ovalis*

(I) Preliminary research with inhibitors

By Toshinobu ASAI and Seiichi NASUNO

Division of Physiology.

D-glucuronic acid was found to be adaptively oxidized by *Pseudomonas ovalis* 40F. Enzyme systems concerning the metabolism of D-glucuronic acid by the use of some inhibitors. From the inhibition with fluoride, monoiodeacetate, arsenite, 2,4-dinitrophenol and azide, it was presumed that dehydrogenase systems containing DPN or flavin as a coenzyme, and also oxidative decarboxylation and oxidative phosphorylation participate in the oxidation of D-glucuronic acid. Moreover, the existence of succinodehydrogenase was also suggested from the inhibition with malonate to succinic acid oxidation.

Bull. Agr. Chem. Soc. Japan, **22**, (1958) 6–10

On the metabolism of D-glucuronic acid by *Pseudomonas ovalis*

(II) Metabolic pathway

By Toshinobu ASAI and Seiichi NASUNO

Division of Physiology.

From the results obtained by the method of successive adaptation and paper chromatography of reaction mixtures with intact cells, it was presumed that D-glucuronic acid was first converted to 5-keto-L-galactonic acid through D-galacturonic acid and then further oxidized. The accumulation of pyruvic acid was observed in the reaction mixtures when the enzyme system was blocked with sodium arsenite. Furthermore, α-ketoglutaric acid was also found in the reaction mixtures with either D-glucuronic or D-galacturonic acid as a substrate. The resting cells of the the organisms in test showed the ability to oxidize all members of Krebs' tricarboxylic acid cycle, pyruvic and acetic acids. The oxidative pathway of D-glucuronic acid by *Pseudomonas ovalis* was assumed as follows:

D-glucuronic acid⟶D-galacturonic acid⟶ 5-keto-L-galactonic acid ⟶ unknown substances⟶pyruvic acid⟶Krebs' tricarboxylic acid cycle⟶CO_2+H_2O.

Japanese J. Veterinary Sci., Vol. **17**, No. 4, (1955) 145–151

On some cases of malt rootlet poisoning in dairy cattle occurring in Tokyo and Chiba districts

By Hiroshi IIZUKA and Mitsugi IIDA,

Division of Type Culture Collection.

Two cases of food poisoning in dairly cattle were brought under observation in autumn 1954. From the results of the survey performed by us the poisoning cases were believed to be due to the malt rootlet supplied as foodage. The toxicity of malt rootlet was manifested by the same symptoms in a calf (when it had been) fed experimentally. Microorganisms isolated from the deteriorated malt rootlet were *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., *Streptomyces* sp. and *Bacillus subtilis*, but *Penicillium urticae*, which has been said to be the cause of poisoning, was not found in our investigation. We examined the toxicity of cultured broth of these microorganisms by intraperitoneal injection in mice. Cultured broth of *Aspergillus* sp. were found to cause muscular palsy in the injected mice, but the other mold strains, *Streptomyces* sp. and *Bacillus subtilis* did not show any toxicity to mice. The strain which showed the toxicity to mice was identified to be *Aspergillus oryzae* var. *microsporus*.

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